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A Histopathological Study of Multiple Myeloma

By

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(Received for publication, May 12, 1960)

INTRODUCTION

The first case report of multiple myeloma was published by Watson and Bence-Jones in 1845 and since then the disease has been the theme of many studies[1-5]. But the studies on this tumor were mostly centered on morphological or biological researches on the tumor-constituting cells, inquiries into the general pathology of the disease being relatively rare. So, the present authors undertook elucidation of the pathological characteristics of this disease and with the outcome as starting point, attempted a study on its essential nature. The results obtained were as reported in the following.

MATERIALS AND METHOD

As materials, 13 cases of multiple myeloma and 1 cases of plasma-cell leukemia autopsied at the Pathological Laboratory, Tohoku University School of Medicine between January 1954 through June 1959, and a case of plasma-cell leukemia autopsied at the Pathological Laboratory, Fukushima Medical College in 1953, 15 cases in total. These samples were prepared into paraffin blocks and frozen specimens in the routine manner, stained by various methods and subjected to histopathological examination.

RESULTS

I) On the tumorous pathological changes.

1) Changes in the osseous system.

i) Scope of tumorous proliferation.

In this disease, we can observed a systematic tumorous proliferation in the bone system in an incomplete way. The tumorous proliferation and the bone resorption tended to be more active in the flat bones, including the ribs, the sternum, the skull and the pelvis, than in the long bones. Among the long bones, the activities were stronger in the upper than in the lower libms. This tendency of stronger proliferation in the flat bones is well consistent with the opinion of

小島 瑞，亀島義男

Anderson[6] who took the red marrows to be the preferred site of occurrence of this type of tumor.

ii) Mode of proliferation of the tumor.

The tumorous proliferation consisted in nodular proliferation mixed with leukemic diffuse proliferation, in varying ratios according to the case and the site. Among the materials of the present study, nodular growth predominated in 8 cases and diffuse growth took the lead in the other 7 cases including the 2 cases of plasma-cell leukemia. In the nodular proliferation foci, however, distinctly definite boundaries were only rarely to be seen around the nodules, which were more often surrounded by diffuse growth. In some cases of leukemic proliferation, too, the part of dense-packed cells strongly resembled nodular proliferation foci. Unequivocal diffuse growth could be observed only in one of the plasma-cell leukemia cases. The process of tumor cell proliferation is traceable by silver staining. Argyrophil fibres are scarcely observable among the nodules and the general appearance is that of a cancer. As the nodules begin to proliferate infiltratively into the surrounding tissues, the argyrophil fibres come to show gradual increase, and often the individual tumor cells are eventually entwined in fine argyrophil fibres.

iii) Destruction and resorption of bone tissues.

Pictures of destruction and resorption of bone tissues were apparent in nearly all the cases. By grades, these could be classified into 7 severe-graded cases, 4 moderate-graded cases, into 3 mild-graded cases and 1 nearly negative case (of plasma-cell leukemia). The frequency of pathological fractures of bones is given by Bayrd and Heck[7] as 16%, by Carson *et al.*[8] as 55% and by Snappers *et al.*[9] as 62%. Among the present authors' subjects, 9 cases (60%) showed such fractures, most frequently in the ribs (5 cases), next in the vertebrae (4 cases), in the sternum and the ulna in 1 case each. Multiple pathological fractures in the same case were frequent in the ribs, and in the vertebrae the fracture preferentially came forth in the lower thoracic or lumbar vertebrae, involving paraplegia and in turn causing ascending pyelonephritis.

In this disease, the extraordinarily active resporption of the bone tissue in spite of the not very intense destructive proliferation, less intense than in other malignant tumors, and the absence of reactive osseous or osteoid formation in the site of bone resorption are probably very specific findings of this disease.

iv) Myeloma cells.

The cells constituting myeloma show rather wide morphological variability by cases and by sites, but after all, they belonged to the so-called plasma-cell type myeloma in all our cases. The tumor cells varied rather notably in size but their nuclei usually stood eccentric, were rich in chromatin, were nearly always found containing nucleoli under methylgreen-pyronin staining and their

protoplasma were pyroninophil. Russel's bodies were very rare, but often violet granules were found accumulated under Weigert's fibrin staining. Bi- or trinuclear cells were rather frequent and on some occasions polynuclear cells with half a dozen nuclei were observable. Nuclear division was detected far less frequently than anticipated, and abnormal division of nuclei could not be definitely demonstrated.

v) Intercellular substances in the tumor.

Varying quantities of argyrophil fibres and collagenous fibres could be found among the tumor cells. Besides, the stroma contained more or less large accumulations of eosinophilic amorphous substances and comparatively rarely of bleeding. The amorphous substances stained red-violet or violet by Weigert's fibrin stain, was pyroninophil under methylgreen-pyronin staining, stained red under PAS staining and sometimes showed metachromasy under gentiana-violet staining, but only in low degree. Such amorphous substances were relatively scanty in the sites of active tumor-cell proliferation but could be often found in the herds of tumor cells more or less advanced in degenerative process. Such tumor cells in degenerative process gradually come to show staining behaviors similar to the amorphous substances above and in new necrotic foci, the amorphous substances take the form of granules. In such a form, these substances show semblance to the necrotized and destroyed cell bodies, so that it may be inferred that at least a part of the intercellular amorphous substances originate in the tumor cells themselves. These amorphous substances probably flow about in random direction within the tumor tissues, taking the form of myelin, and often encircling the blood vessels, show circular formation. Sometimes, the picture of their penetrating through the vascular walls and invading the lumina.

2) On the extramedullary dissemination of the tumor.

i) Continuous extention of the tumor

The tumor tissues outside the bone-marrows were mostly found around the bone. These places nearly correspond to the sites where bone trabeculae were strongly destroyed by intramedullary tumor tissue, or to the pathologically fractured sites. And most of the tumor tissues around the bone have macroscopically evident nodules, sometimes showing a lump of tumor of the size of a baby's head. On the other hand, the tumorous proliferation around the bone rarely takes place at the sites where the tumor has the tendency to diffuse proliferation.

ii) Distant metastasis.

The distant metastasis of this tumor was found in 9 cases (60%) among 15, and it was found mostly in the liver (6 metastatic cases), the spleen (7 metastatic cases) and in the lymph nodes (7 metastatic cases) as Churg & Goldon[10] and Hino[11] pointed out. Besides, also in the testis, the subcutaneous tissues, the

muscles, the dura, the kindney and the suprarenal body etc. metastatic foci were observed. This distant metastasis of the tumor, differing from its continuous extension, is seldom observed by macroscopic examination, but mainly found by histological research. Myelomatic cells appeared in peripheral blood in 12 cases (86%) among the 14 cases examined. The frequency of their appearance was usually below 10% of the lecoucyte count, except in the two cases of plasma-cell leukemia.

II) On the pathological changes based on paraprotein.

In the lymph nodes: Perceptible swelling was not at all seen in any of the case examined, the lymph nodes being rather atrophied. The chief changes in this part are sinusreticulosis and especially the proliferation of reactive reticulum cells at the marginal sinus.

In the liver: A degenerative picture of the parenchyma of liver was seen only in 1 case. The Kupffer's stellate cells within the sinusoid were generally swollen, but their reaction process against paraprotein was not distinct because of the large quantity of hemosiderin they had taken up.

In the spleen: Except the cases in which metastasis of the tumor was found, the spleen was atrophied and the lymph follicles were remarkably reduced. The central arteries were in most cases thickened and especially their inner walls displayed fibrous or hyaline degeneration and thickened. These sites show the same attitude against stains as the tumorous intercellular amorphous substance. In the medullary cord the multiplication of round reticulum cells and reactive plasma cells was often observed together with the increases of the fiber elements.

In the kidney: Usually the kidney had gained a little in weight, and has a peculiar luster. In three cases the kidneys suffered from either pyelonephritis or renal abscess. It was only in 1 case that the infiltration of tumorous cells into the kidney was observed. The change in the glomerules is usually mild, as Allen[12] has stated, consisting only in swelling or in slight thickening of the basal membrane. In the urinary tubules, particularly in the lower ones, a great many protein casts were not seldom. The staining behavior of these casts was similar to the above-mentioned intercellular eosinophilic substance in the tumor, but the stainability of the casts themselves differed not a little owing to the difference of their location within urinary tubule and of the stratified construction which they often present. The tubular epithelium near the casts began to muliply actively as soon as they fell into destruction and degeneration, forming polynuclear cells resembling (foreign giant cell) around them. The urinary tubules, particularly the upper ones, were not seldom atrophied or swollen edematously. In the stroma, an invasion of eosinophilic substance and infiltration of cells were observed. Bell[13] stated that he saw protein casts in 7 cases among 11,

while the present authors saw them remarkably formed in 6 cases, moderately in 2 cases, and slightly or negatively in 8 cases.

In the lung: The walls of the interlobular blood vessels in the lung were more or less swollen and expanded, sometimes showing fibrous degeneration. In some cases the lumen was remarkably narrowed on account of the thickening of the blood vessel wall. The same change was found also in the alveolar wall: An amorphous substance that shows the same staining behavior with the previously described intercellular amorphous substance in the tumor was found accumulated in the alveolar walls and flowing out into alveolar lumens often to form hyaline membranes. Also the alveolar walls were found fibrously thickened and in the sites where they ware swollen nodularly they sometimes take the form of Masson's body. Where the pulmonary stroma was fibrously thickened the lung tissue formed irregular atelectasis, and in the other regions compensative emphysema was observable. Often the exsudative fluid that stained comparatively light was reserved in the alveolar lumen, and such an exsudation was highly accelerated by infection. In this case the frequency of complication with pneumonia is known to be high as Snapper[9] etc. indicated, and also in this present research the complication was seen in 8 cases (53%). Thus, the exsudation is widespread even in the foci in which cellular infiltration is mild.

III) On the complicated pathological changes.

Calcification was seen in the kidney (14 cases), the lung (8 cases) and the liver (3 cases) etc. and was generally mild except in two cases of calcification in the lung. The degree of the calcification did not necessarily have a definite relation with that of destruction or resporption of bone.

Hemosiderosis was chiefly found in the liver and the spleen, especially severely in the latter. Seeing that the hemosiderosis was the severer, the larger the quantity of transfusion, this is deemed to be due to transfusion.

DISCUSSION

It is known that there are cases in which leukemic diffuse growth is seen besides the cases that show nodule formation, in the tumorous cell proliferation in multiple myeloma (Heilmeyer)[14]. According to the findings of this present studys, it is rare that a case shows either nodular growth or diffuse proliferation alone, for both the types of proliferation are found mixed in most cases. Then, why the nodular growth and the diffuse proliferation are observable in the proliferation of this tumor? Though scarcely any explanation has been presented as yet on the cause and reason, the present authors are inclined to find the answer in the nature of the tumor cells. The reason shall be stated in the

following.

Though many theories are being presented with regard to the form of tumor cells, they may be roughly divided as follows:

1. Plasma-cell theory (including that of undifferentiated cells).
2. Blood parenchyma-cell theory (of myeloblasts, myelocytes, lymphocytes, erythroblasts etc.).

The blood parenchyma-cell theory was predominant while the form of the tumor cell had been observed by tissue specimen, but by the advance of hemato-cytological research, especially with the prevalence of sternal puncture the plasma-cell theory, first advocated by Wright[15] and supported by Diggs *et al.*[16] and Bayrd *et al.*[17], came to be regarded with great importance. As already stated, all the cases the present author studied were of the plasma-cell type myeloma. Although there are many theories such as the blood-cell theory[18], the reticulum-cell or the reticuloendothelium theory[17),19] and the theory of transformation of blood-vessel adventitial cells[20] as for the origin of the plasma cell, yet no convincingly definitive conclusion was obtained so long as the present authors' experiments and researches go. Only, seeing the facts that this tumorous proliferation was confined within the bone tissues and that extra-medullary proliferation was remarkably milder than the former proliferation, it will safely be concluded that the mother-cells of this tumor exist in the medullary tissue. However, whether this cells are still in the state of blood cells or of tissue cells, the opinions are still divergent. The present authors should rather like to regard the controversy to be a direct reflexion of the nature of the plasma cells. That is, the plasma cells are considered originally to have both the characters of blood cells and tissue cells. If the matter is viewed from this point, it will easily be understood that the nodular growth and the diffuse proliferation do appear simultaneously in the process of this tumorous proliferation. The tumor cells and the mode of proliferation of the 13 cases of multiple myeloma and the 12 cases of plasma-cell leukemia were studied and it was found that the two are intrinsically the same disease, leading to the following inference: in plasma-cell leukemia the blood cell character alone is emphasized out of the two characters of the tumor cells, while in the multiple myeloma both the two characters are mixed positively in various ratios.

The other greatest characteristics of this tumor are the invasion of paraprotein into each organs that appears accompanied by the tumorous cell proliferation, and the tissue reaction following it. Infering from histological researches, a certain substance released by the secretion or destruction of the tumorous cells, entering directly or through the lymph way into the blood road, seemed to cause the paraproteinemia. Pathological renal changes are well known as pathological changes cuased by paraproteinemia, but looking through the present authors'

findings pathological pulmonary changes should also be considered seriously as well as the pathological renal change. In the kindney, the reaction based on the appearance of paraprotein substance in the urinary tubules layed the leading part. As far as the authors' findings tell, the frequency of the appearance of Bence-Jones protein bodies in urine seemed to go parallel with the degree of the pathological renal change. A certain correlation between the protein content of serum and the pathological renal changes, however, could not be found. In the lung, it was ascertained that paraprotein was accumulated on the alveolar walls owing to the disturbance of the blood vessel system, then it flowed out into the lumina in which it was gradually organized as long as the flowing continued, causing peculiar atelectasis and compensatory emphysema.

CONCLUSION

The author made histopathological researches of 13 cases of multiple myeloma (plasmacytoma) and 2 caes of plasma-cell leukemia and obtained in summary the following results.

1) The proliferation of this tumor is confined solely in the bone-marrows and, though inperfectly, is systematic.

2) Nodular proliferation and diffuse proliferation were seen in the process of proliferation of this tumor. These are considered to be based on the characters of plasma cells which have the two characters, of blood cells and of tissue cells.

3) The destruction and the resorption of bone by the invasion of tumor are exceedingly remarkable, while reactive bone proliferation is absent.

4) Appearance of eosinophilic amorphous substance accompanying the proliferation of argyrophil fibres and the destruction and necrosis of cells are observed among the tumor cells.

5) The tumor cells bear closest resemblance form to intra-medullary plasma cells. Their origin, however, is not clearly explained by the authors' present researches.

6) The dissemination of the tumor cells towards outside the bone marrows is mainly consists in continuous extension, preferrably forming nodes around the bone. The tumor cells are detected in the peripheral blood in most casses though in a small scale, and sometimes form relatively localized metastatic foci in the liver, the spleen, the lymph nodes, the testes, the subcutaneous tissue and the muscles etc.

7) The pathological change caused by paraprotein is remarkable in the blood vessels of the whole body, especially, in those of the kidney and the lung. a) In the kidney, the chief changes are the formation of protein casts inside the urinary tubules and the reaction accompanying it. b) In the lung, the invasion of paraprotein into the alveolar walls and the alveolar lumens, and the formation of peculiar atelectasis and compensatory emphysema due to the variation and

the organization of the paraprotein are observed.

8) Besides, calcification and hemosiderosis are occasionally noticed as complicating pathological changes.

References

1) Kahler, O., Prag. med. Wschr, 1889, **14**, 45.
2) Geschickter, C.F. & Copeland, M.M., Arch. Surg., 1928, **16**, 807.
3) Lichtenstein, L. & Jaffe, H.L., Arch. Path., 1947, **44**, 207.
4) Wurmann, F. & Wunderly. C., Die Eiweisskörper des Menschen. 2 Aufl. Benno. Schwabe Basel, 1952.
5) Sugano, H., Acta path, Jap., 1959, **9**, 217.
6) Anderson, W.A.D., Pathology. 3 ed. St. Louis, Mosby, 1957.
7) Bayrd, E.D., & Heck F.G., J.A.M.A., 1957, **133**. 147.
8) Carson, C.P., Ackerman, L.V. & Maltby, J.D., Amer. J. Clin. Path., 1955, **25**, 849.
9) Snapper, I., Turner, L.B. & Moscowitz, H.L., Multiple Myeloma. Grune & Stratton, 1953.
10) Churg, J. & Gordon, A. J., Amer. J. Clin. Path., 1950, **20**, 934.
11) Hino, S., Gan no Rin., (Japanese) 1955, **1**, 531.
12) Allen, A.C., The kidney, medical & surgical disease. Grune & Stratton, 1951.
13) Bell, E.D., Amer. J. Path., 1933, **9**, 393.
14) Heilmeyer, L. & Begemann, H., Handbuch der inneren Medizin, 2 Bd. Blut & Blutkrankheiten. 4 Aufl. Springer, Berlin, Heidelberg, 1951.
15) Wright, J.H., referred to 9) Snapper *et al.* Multiple Myeloma, N.Y. Grune & Stratton, 1953.
16) Diggs, LW. & Sirridge, M.S., J. Laborat. Clin. Med., 1947, **32**, 167.
17) Bayrd, E.D., Blood., 1948, **3**, 987.
18) Naegeli, O., Blutkrankheiten & Blutdiagonstik. 5 Aufl. Berlin, Springer, 1931.
19) Rohr, K., Das menschliche Knochenmark. Georg Thieme, Leipzig. 1940.
20) Amano, S., Acta haem. Jap., (Japanese) 1946, **9**, 25.
21) Apitz, K., Virchows Arch., 1940, **306**, 631.

CAPTIONS OF PLATES

Plate I. Smear specimen of bone-marrow puncture: The tumor cells are almost round and comparatively small. The nucleoli are conspicuously large. The protoplasm is coarse and comparatively little in quantity. (Giemsa staining).

Plate II. Histological picture of the femoral marrow: The tumor cells are growing up into nodular shape. (H–E staining).

Plate III. Histological picture of the sternal marrow: The tumor cells are proliferating leukemically and diffusely. (H–E staining).

Plate IV. Histological picture of the sternal marrow: The tumor cells, infiltrated into the osseous substance, are actively destroying and resorbing the osseous substance. Reaction of the bone tissue against them is not seen at all. (H–E staining).

Plate V. Histological picture of a metastatic herd of the left hypogastric tumor: Conspicuous is the grotesque figure of the necrotic substance which is in the shape of myeline or wheel. The central part of the wheel-shaped constructions is mostly taken up by the lumens of blood vessels. (H–E staining).

Plate VI. Histological picture of a tumor in a rib: The increase of the argyrophil fibres is remarkable. Some of them are connected with one tumor cell each. (Pap's silver staining).

Plate VII. Histological picture of a tumor in the liver: Tumorous nodules are seen in the lobules. (H–E staining).

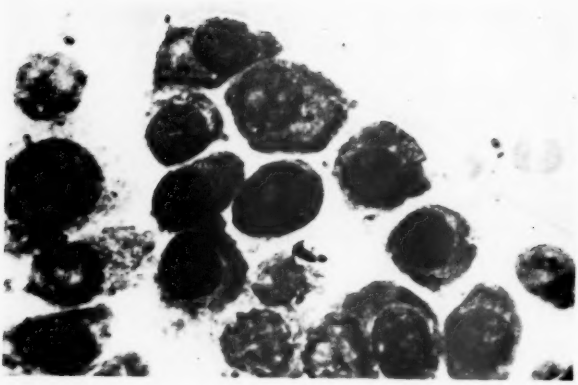
Plate VIII. Histological picture of a urinary tubule: The protein casts inside the tubule condensed into a lump, cells of a part of the tubular epithelium round the lump becoming giant cells. In the urinary wall in that part destruction of the epithelial cells is seen. (H–E staining).

Plate IX. Histological picture of the lung: The alveolar wall is nodulatedly thick on account of the pile of amorphous substanec. (H–E staining).

Plate X. Histological picture of the surroundings of pneumonia: Evident invasion of paraprotein into the alveolar lumina. (H–E staining).

Plate XI. Histological picture of the central artery of the spleen: The arterial wall is remarkably thickened and the invasion of paraprotein is noticeable in its wall. H–E staining).

Plate XII. Histological picture of the lung: Conspicuous calcification of the alveolar wall. (H–E staining).



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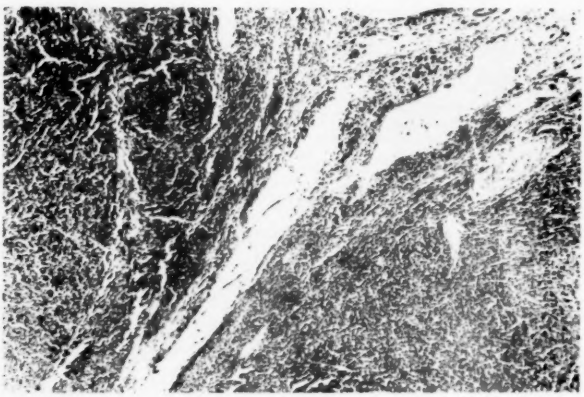

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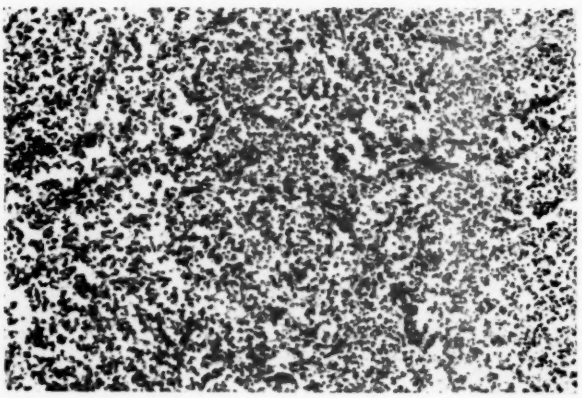

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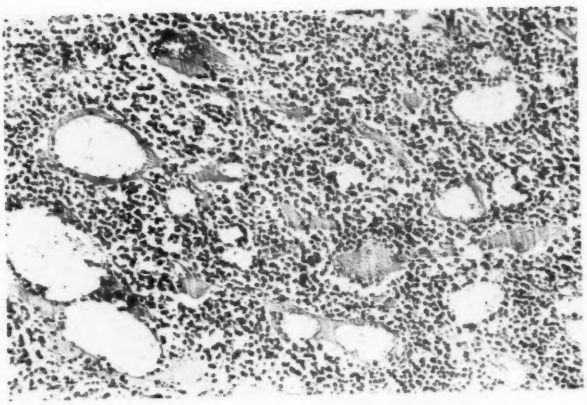

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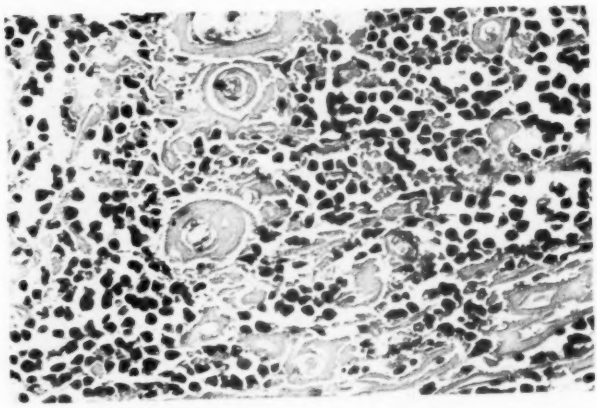

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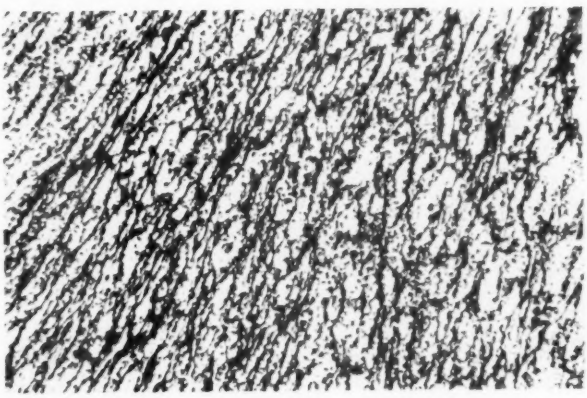

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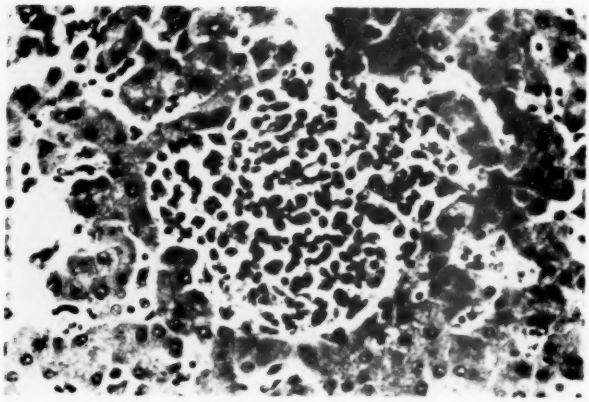

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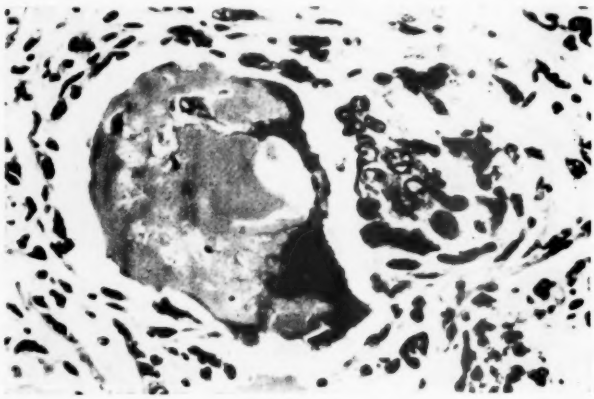

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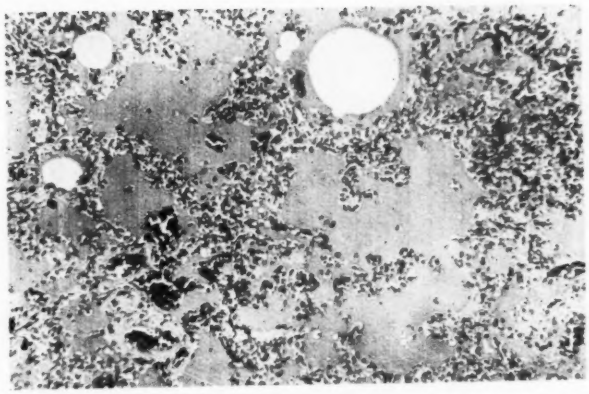
Plate IX



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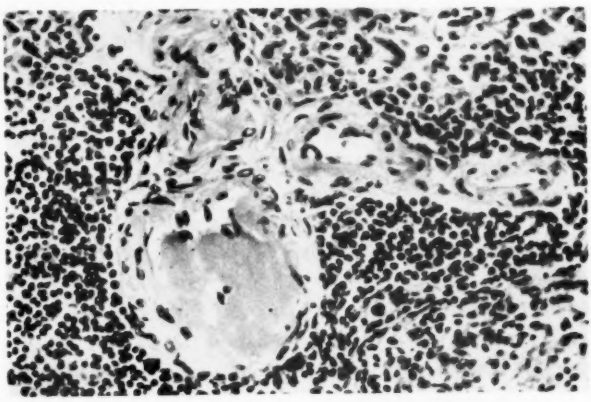

Plate XI

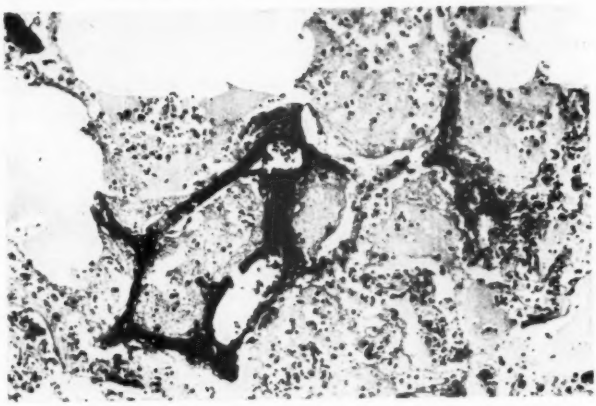

Plate XII

Tohoku J. Exper. Med., 1960, 72, 322-327

The Labelling of Influenza Virus with Radioactive Phosphorus in *Maitland* Type Tissue Culture

First report, Growth Characteristics of Myxoviruses in Tissue Culture

By

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(Received for publication, May 12, 1960)

A variety of studies performed with bacterial viruses, both on the penetration and mutliplication mechanism have indicated the usefulness of radioisotope technics, in pursuing these series of events in a fairly accurate manner. They are conducted one by preparing radioactive virus or radioactive host cell, the other by analyzing the uptake of radioisotope by the infected cell from the medium. Radioisotopes thus introduced into the system ended up as some host or viral components.

With animal viruses, however, the problem still remains in labelling procedure of virus particle itself, in spite of numerous reports already appeared[1)-8)]. The specific activity of these viruses was fairly low, thus the fact did not allow full description of the fate of a virus inside the cell. One more problem which is particularly the case with influenza viruses is the fact that host components hardly discernible from viral components are often found with the purified virus particles. These components should be eliminated more in elaborated manner.

Having these kind of considerations in mind, Maitland type tissue culture has been employed for the labelling of influenza virus with radioactive phosphorus, using chicken chorioallantoic membrane as the host cell. Purification procedure of the virus was also exmained to eliminate normal components and non-viral radioactive phosphorus.

Thus the present paper chiefly concerns on the fundamental technic itself. Final product revealed the high specific activity in a sense of counts per minute per hemagglutinin (cpm/HA).

MATERIALS

Virus: The PR8 strain of influenza A virus, having undergone seven passages in ferrets, 593 passages in mice, 170 passages in embryonated eggs, 40 to 41

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passages in mice and one passage in embryonated eggs was used throughout the experiments.

Tissue: Chorioallantoic membranes (CAM) obtained from 15-day-old embryonated eggs were used as host cells. Hanks' solution from which all phosphorus was removed was employed as a medium.

P^{32} and determination of radioactivity: Radiophosphorus in the form of orthophosphate in diluted HCl supplied from the Radiochemical Center, Amersham, England was used. All determinations of radioactivity were carried out, as a rule, in duplicate by evaporating 0.1 ml of the preparations on planchets and counting the activity with a mica end-window Geiger-Müller tube[9]. In the text, radioactivity was expressed as the counts per minute (cpm) in 1 ml amount of examined specimens.

EXPERIMENTAL

Production and purification procedure of labelled virus

Firstly, chorioallantoic membranes of 15-day-old embryonated eggs were removed and washed three times with sterile saline, followed by blotting with filter paper. For Maitland type culture, usually 100 ml amount of Erlenmeyer flask was used. 11.5 ml of Hanks' solution containing desired amount of P^{32} was distributed in each flask with five pieces of chorioallantoic membrane. 1.5 ml of a 10^{-1} dilution of infected allantoic fluid was added as the inoculum. These flasks were incubated at 37°C for 24 hours under shaking condition. After 24 hrs of incubation, fluids were collected aseptically and were obtained as transparent supernatant after the centrifugation at 3,000 rpm for 15 minutes.

The supernatant thus obtained was cooled in an ice bath and 1/10 volume of 10% supension of sterile chicken red blood cells which were previously chilled were added. They were left in an ice bath for two hours with occasional shaking and the mixtures were centrifuged to obtain the virus adsorbed red cells. These cells were washed four times with M/100 phosphate buffered saline of pH 7.0 (PBS) which had been previously chilled and equal to the amount of original viral material. The sediment was then resuspended in 2/3 the original volume of PBS containing antibiotics and incubated with frequent gentle shaking in a water bath of 37°C for 90 minutes. At the end of this elution process, the cells were removed by low-speed centrifugation at 3,000 rpm for 15 minutes. The supernatant (referred as the lst eluate) was then subjected to the same adsorption-elution procedure as described above, except that the washed cells were suspended in 2/3 volume of the lst eluate. At the end of the elution period, the cells were removed by low-speed centrifugation at 3,000 rpm for 15 minutes and the supernatant (referred as the 2nd eluate) was then ultracentrifuged to precipitate the virus particles. After ultracentrifugation at 26,000 g for one hour, the supernatant was discarded and the pellet was resuspended in 1/4 the original

TABLE I. Purification Procedure of the PR8 Virus Labelled with P^{32}

① 5 CAM of 15-day old embryonated eggs
② 11.5 ml of phosphorus free Hank's solution containing desired amount of P^{32}
③ 1.5 ml of a 10^{-1} dilution of infected allantoic fluid

↓ in 100 ml Erlenmeyer flask with cotton plug

↓ incubated at 37°C for 24 hours under shaking condition

↓ infected fluid

↓ centrifuge at 3,000 rpm for 15 min.

↓ supernatant

↓ add 1/10 vol. of a 10% suspension of chicken red cells and stored at 0°C for 2 hours

↓ low centrifugation

↓ sedimented red cells

↓ washed four times with M/100 PBS of pH 7.0

↓ sedimented red cells

↓ eluted with 2/3 the original vol. of PBS at 37°C for 90 min.

↓ centrifugation at 3,000 rpm for 15 min.

↓ supernatant (1st eluate)

↓ repeat the cycle of adsorption to and elution from red cells followed by repeated washing and elution with PBS, 2/3 volume of the 1st eluate

↓ centrifugation at 3,000 rpm for 15 min.

↓ supernatant (2nd eluate)

↓ ultracentrifugation at 26,000 g for 1 hour

↓ sediment

↓ resuspended to 1/4 the original vol. of PBS

↓ centrifugation at 3,000 rpm for 15 min.

↓ supernatant (spinco-suspension)

↓ dialysed against 2–3 l of PBS

↓ centrifugation at 3, 000 rpm for 30 min.

↓ Purified P^{32} labelled virus

volume of PBS containing antibiotics, then the suspension was again centrifuged at 3,000 rpm for 15 minutes. Finally, the supernatant (referred as Spinco-suspension) was placed in a cellophane bag and dialyzed against two to three liters of PBS for a few days at 4°C. The dialyzed specimen was centrifuged at 3,000 rpm for 30 minutes. Thus, the final preparation of the purified labelled influenza virus was obtained and ready for study. Flow sheet of these procedures are illustrated in Table I.

Assay of the final preparations

According to this technic, a number of experiments were repeated for a period of six months. Firstly, to increase the specific activity of the final preparation, repeated experiments were performed to determine the amount of P^{32} to

TABLE II. Properties of Labelled Virus Preparations

Preparation*	HA/ml	cpm/ml†	cpm/HA‡
50 μc–virus	256	195	0.76
100 μc–virus	512	963	1.88
150 μc–virus	1,024	4,120	4.02
200 μc–virus	1,510	12,410	8.22

* P^{32} in a concentration of 50 μc, 100 μc, 150 μc and 200 μc per piece of CAM was used respectively.

† The radioactivity is expressed as counts per minute (cpm), corrected for 1 ml volume.

‡ Specific activity is expressed as counts per minute (cpm)/ hemagglutinin titers (HA).

TABLE III. Purity of Labelled Virus Preparations on the Basis of Two Criteria

Preparation	(A) Counts found with starting material	(B) Counts in the supernatant after adsorption to chicken red cells*	(C) Counts in the supernatant after ultracentrifugation†	% of non-viral P^{32} (B/A)	(C/A)
50 μc–virus	47	4	5	8.5	10.6
100 μc–virus	250	16	22	6.2	9.0
150 μc–virus	1020	52	58	5.1	5.7
200 μc–virus	3110	143	165	4.6	5.3

* Four ml of 1% suspension of chicken red cells was packed and mixed with 1/4 dilution of labelled virus preparations in PBS. After occasional shaking at 0°C for 90 min., the mixtures were centrifuged and the radioactivity of the supernartants was measured.

† One fourth dilution of labelled virus preparations in PBS was centrifuged at 26,000 g for 1 hour. The radioactivity found in these supernatants was illustrated.

be incorporated into the medium. One of the typical experiments was shown in Table II, where the concentration from 50 to 200 μc per piece of chorioallantoic membrane was examined. Purification of the virus was made from respective harvest. Finally with purified materials, both hemagglutinin and radioactivity were examined. It can be seen from the Table that under the condition employed here, the shift of specific activity (cpm/HA) was parallelled with the increase of P^{32} put into the medium. As a result, specific activity of 8.22, which will be the highest titer shown among related experiments, was obtainable. However, one has to also notice the low EID_{50}/HA which is usually the case with the harvest of 24 hours in this culture. The harvest usually revealed the tier of 1024 hemagglutinin per ml by means of Salk's pattern test where EID_{50} ranged from 8.0 to 8.5. Secondly, purity of the final preparations was examined in two different approaches. One was made by adsorbing the preparation with red cells and determining the residual radioactivity of the supernatant. Four ml of one

per cent suspension of chicken red cells were packed and mixed with 1/4 PBS dilutions of labelled virus preparations. After occasional shaking at 0°C for 90 minutes, the mixtures were centrifuged and the radioactivity of the supernatant was measured. Alternative way to test the amount of contaminating non-vial P^{32} was carried out by ultracentrifugation. One fourth dilution of labelled virus preparation in PBS was centrifgued for one hour at 26,000 g. The radioactivity left in the supernatant was calculated and taken as impurities.

Table III shows the results obtained with four preparations. Percentage of the impurities thus ranged from 4.6 to 10.6. However, high specific virus preparation was obtained in a culture incoroporated with 200 μc of P^{32} and this preparation revealed the least contamination so far.

DISCUSSIONS

Elucidating the mechanism of any viral infections, particularly on cellular level, only a limited amount of seed virus can be employed if multiple infecton to host cells is to be avoided. Thus the preparation of high specific activity is always in need for carrying out such a experiment. Literal consideration seems to reveal that the grade of labelling of influenza virus with radiophosphorus is largely dependent upon the experimental system employed for their reproduction. Maitland type tissue culture was examined from such a reason and as a result, high specific and less contaminated virus was obtainable here. Moreover, these experiments were repeated several times and the results were always reproducible. Thus the paper chiefly concerned on the technic itself, rather than theoretical considerations.

By using deembryonated eggs as the host, influenza virus of higher specific activity has been already obtained than in the allantoic cavity of the embryonated eggs[5]. However, virus production was considerably low in this occasion. Maitland type tissue culture was not only favourable in this sense, but also simpler in performing the experiment. Labelling of the virus in dispersed cell culture was recently successful with poliovirus[10], Newcastle disease virus[11] and fowl plague virus[12]. But at least up to present, no suitable cells for the PR8 virus growth have been described, as has been reported here.

At the end of this discussion, however, one more comment should be added on the use of $AlPO_4$ as the adsorbent in the purification procedure of influenza viruses. Recently, we have been successful in purifying the Sendai virus by means of $AlPO_4$ gel.[13-14] This procedure was apparently preferable to the use of red cells as far as its potentiality in removing host components are concerned, no matter how the contamination of inorganic phosphorous is unavoidable. When the labelled Sendai virus thus prepared was examined by Ouchterony's agar gel diffusion technic in addition to the aforementioned two

criteria, the advantage of $AlPO_4$ in removing the hots components was apparently revealed. Details of these results will be reported elsewhere[15].

SUMMARY

In labelling the PR8 strain of influenza virus with radioactive phosphorus, Maitland type tissue culture was employed. Here a piece of chorioallantoic membrane was used as the host cell in Hanks' solution. In this system, P^{32} put in a medium, from which non-radioactive phosphorus was removed previously, was highly incorporated into the virus particles and as a result as high as 8.22 specific activity in a sense of cpm/HA was obtainable. In purifying the labelled virus, adsorption to and elution from chicken red cells together with ultracentrifugation was employed and a large amount of non-viral phosphorus was removed. The advantage of the technic described here will be in its reproducibility in preparing high specific virus. However, EID_{50}/HA ratio was somewhat low with obtained specimens.

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References

1) Graham, A.F. & McClelland, L., Nature, 1949, **163**, 949.
2) Graham, A.F. & McClelland, L., Can. J. Res. E., 1950, **28**, 121.
3) Ward, T.G., Am. J. Hyg., 1950, **52**, 107.
4) Hoyle, L., Jolles, B. & Mitchell, R.G., J. Hyg., 1954, **52**, 119.
5) Liu, O.C., Blank, H., Spižizen, I. & Henle, W., J. Immunol., 1954, **73**, 415.
6) Hoyle, L. & Frish-Niggemeyer, W., J. Hyg., 1955, **53**, 474.
7) Liu, O.C., Trans. N. Y. Acad. Sci., 1956, **18**, 249.
8) Hoyle, L. & Finter, N.B., 1957, J. Hyg., **55**, 290.
9) Ishida, N., Kosaka, Y. & Sasaki, Y., Tohoku J. Exp. Med., 1959, **71**, 151.
10) Taylor, J. & Graham, A.F., Virology, 1958, **6**, 488.
11) Franklin, R.M., Rubin, H. & Davis, C.A., Virology, 1957, **3**, 96.
12) Wecker, E. & Schäfer, W., Z. Naturforschg. 1956, **116**, 181.
13) Miller, H.K, & Schlesinger, R.W., J. Immunol., 1955. **75**, 155.
14) Frommhagen, L.H. & Knight, C.A., Virology, 1959, **8**, 198.
15) Kudo, H., Onodera, T. & Ishida, N., to be published

Tohoku J. Exper. Med., 1960, 72, 328–337

Molisch-positive Mucopolysaccharides of Gastric Cancers as Compared with the Corresponding Components of Gastric Mucosae

Fifth Report: On MPSs (Mucopolysaccharides) III*

By

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In the First Report[1] of this series, we reported the fractionation and isolation of four kinds of Molisch-positive mucopolysaccharide (MPS I–IV) from gastric cancer tissue and gastric mucosa. The physical, chemical and biological properties of the two kinds of group active mucopolysaccharide, MPSs I[2] and IV[3] as well as those of group inactive MPSs II[1], were reported in succession.

This paper deals with "MPSs III" of gastric cancer tissue and of gastric mucosa which have been prepared by somewhat modified procedure of that described in the First Report[1].

EXPERIMENTS AND COMMENT

Preparation Procedure

Preliminary experiments indicated the group inactivity of MPSs III, so that starting fractions, **Ppt″$Ba(AcO)_2$ 20~40**,[1] from cancerous and non-cancerous tissues were combined respectively according to their sources regardless of their blood groups.

1) Cancerous substance

a) Combined **Ppt″$Ba(AcO)_2$ 20~40** was subjected to fractional precipitation with Na_2SO_4 same way as previously described. The precipitate which appeared at Na_2SO_4 concentrations between 20 and 30% (w/v) was dissolved in water and dialyzed against running water for two days. Then, without the treatment with ion exchange resins in original procedure, it was condensed *in vacuo* to syrupy consistency, precipitated by addition of ethanol, washed with

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* 231st report of Masamune and co-workers' "Biochemical Studies on Carbohydrates".

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ethanol and ether and dried – **Ppt′20-30%Na_2SO_4**.

b) 1.8 g. of **Ppt′20-30%Na_2SO_4** were taken up in 150 ml. of 2% $Ba(AcO)_2$. The yellowish turbid solution (pH 6.6) was stood for 30 minutes in ice-water, then centrifuged (53,800 x g; 45 minutes) in a preparative ultracentrifuge (Type 40P, Hitachi Ltd.). The centrifugate was washed with ethanol and ether and dried. A white powder weighing 600 mg. was obtained – **Ins‴$Ba(AcO)_2$**. To the yellowish clear supernatant chilled on ice was added ethanol dropwise under constant stirring, and the resulting precipitates at six ethanol concentration ranges were separated by centrifugation (53,800 x g; 45 min.) immediately after ethanol concentrations reached to those as designed. The precipitates were washed with ethanol and ether, then dried in a desiccator to give following fractions:

Ppt‴$Ba(AcO)_2$- 0∼10*	Yield	55 mg.
Ppt‴$Ba(AcO)_2$-10∼20		39 mg.
Ppt‴$Ba(AcO)_2$-20∼25		56 mg.
Ppt‴$Ba(AcO)_2$-25∼30		132 mg.
Ppt‴$Ba(AcO)_2$-30∼40		890 mg.
Ppt‴$Ba(AcO)_2$-40∼50		162 mg.

* Numbers represent ethanol concentrations at which the indicated precipitates were obtained.

The mother fluid of **Ppt‴$Ba(AcO)_2$-40∼50** was concentrated *in vacuo* to about 50 ml., dialyzed against running water for 24 hours, distilled again *in vacuo* to a syrup, precipitated with ethanol, washed with ethanol and ether and desiccated—**Supern‴$Ba(AcO)_2$-50**. Yield 38 mg.

The fractions, from **Ins‴$Ba(AcO)_2$** up to **Ppt‴$Ba(AcO)_2$-20∼30**, gave positive Goldschmiedt reaction, but the others gave negative Goldschmiedt and strongly positive Molisch reaction.

c) 830 mg. of **Ppt‴$Ba(AcO)_2$-30∼40** were dissolved in water. The resulting solution (yellowish clear, pH 6.8) was passed through 1×15 cm. columns of Amberlite IR 120 (H^+) and of IR 45 (OH^-) in turn. The resins were washed with 50 ml. of water. The yellowish clear effluent of pH 2.0 and washing were combined and lyophilized. 750 mg. (0.11% of the starting dry tissue material) of a fluffy powder were obtained — **cancerous MPS III**.

2) Noncancerous substance

a) Starting fraction, noncancerous **Ppt″$Ba(AcO)_2$-20∼40**, was treated as described in 1) a) and the main fraction, **Ppt′20-30%Na_2SO_4**, was obtained.

b) 1.45 g. of **Ppt′20-30%Na_2SO_4** were fractionated similarly as in 1) b). The fractions prepared and their yields were as follows: **Ins‴$Ba(AcO)_2$** 120 mg., **Ppt‴$Ba(AcO)_2$-0∼25** 912 mg., **Ppt‴$Ba(AcO)_2$-25∼30** 94 mg., **Ppt‴$Ba(AcO)_2$-30∼35** 47 mg., **Ppt‴$Ba(AcO)_2$-35∼40** 81 mg., **Ppt″$Ba(AcO)_2$-40∼50** 336 mg., and **Supern‴$Ba(AcO)_2$-50** 50 mg.

Similarly to the case of cancerous materials, the fractions which precipitated at ethanol concentrations below 30% (v/v) were found to be positive in Goldschmiedt reaction. Among the Goldschmiedt negative and Molisch positive fractions, the yield of $Ppt'''_{Ba(AcO)_2\text{-}40\sim50}$ was found to be the highest, that is, the precipitability of the main fraction differed from that of the cancerous.

c) $Ppt'''_{Ba(AcO)_2\text{-}30\sim35}$, $Ppt'''_{Ba(AcO)_2\text{-}35\sim40}$ and $Ppt'''_{Ba(AcO)_2\text{-}40\sim50}$ were combined, and 400 mg. of which were subjected to zone electrophoresis.

200 mg. of the substance were kneaded with 5 ml. of 0.1 *M* acetate buffer (pH 4.6) and Geon resin[5] (100–200 mesh) to make a paste of adequite consistency. The paste was placed in 1 cm. width at 1 cm. cathodic side from the center of a cell ($34 \times 7 \times 1.5$ cm.) which was previously packed with the supporting medium consisting of Geon resin and the acetate buffer except the space to be applied with the material. The electrophoresis was carried out in a refrigerator at $0\pm2°C$ with current of 30 mA during 44 hours. Then the supporting medium was cut into 34 strips, each 1 cm. width, to each of which were added 20 ml. portions of water. They were stirred thoroughly and allowed to stand for about 30 minutes. 0.3 ml. portions of each fraction were pipetted out from the supernatants and their hexose contents were compared one another by the reaction HR_{10} of Masamune and Sakamoto[6], thus giving the zone-electrophoresis patten which is shown in Fig. 1. Based on this pattern, the fractions were com-

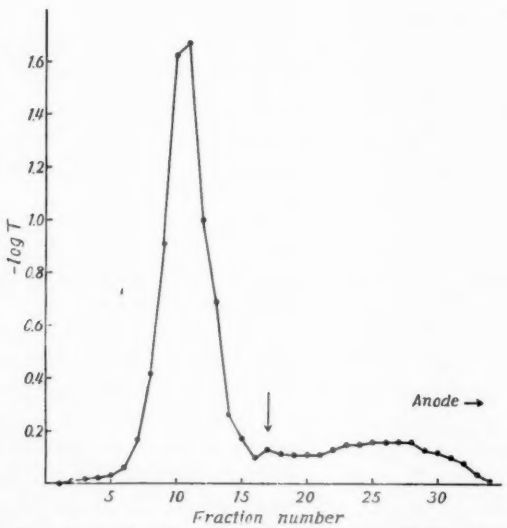
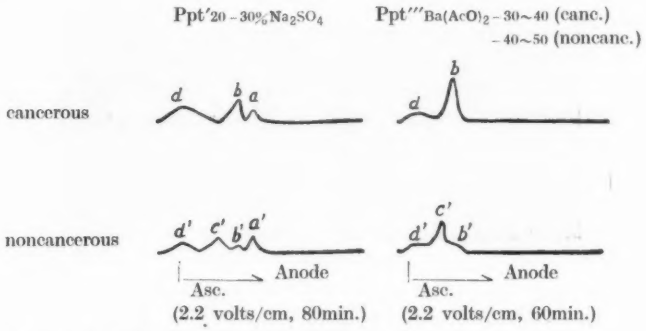


Fig. 1. Zone-electrophoretic pattern of noncancerous $Ppt'''_{Ba(AcO)_2\ 30\sim50}$

bined as to give four main fractions, **Fr. I** (fraction no. 1–7), **Fr. II** (fraction no. 8–12), **Fr. III** (fraction no. 13–16) and **Fr. IV** (fraction no. 17–34). Each main fraction was extracted several times with water and the extract was adjusted to pH 6.6 with 5% NaOH, concentrated under reduced pressure to a small amount, precipitated with ethanol and dried after washing with ethanol and ether. Yields of Frs. I to IV were 8, 87, 25 and 41 mg. respectively.

The other 200 mg. was also subjected to similar operation.

160 mg. of Fr. II were dissolved in 10 ml. of water, shaken with 4 ml. (wet) of Amberlite IR 120 (H^+) and with 3 ml. of Amberlite IR 45 (OH^-) in turn for ten minutes and filtered. The resins were then washed with three portions of 10 ml. water. The slightly yellowish clear filtrate (pH 6.0) and washings were combined and freeze-dried — **noncancerous MPS III**. Yield 133 mg. (0.01 % of starting dry tissue material).



a, *a'* : Goldschmiedt positive substance
b : cancerous MPS III
b' : nocancerous substance which electrophoretically corresponds to canc. MPS III
c' : noncancerous MPS III
d, *d'* : δ-boundary

Fig. 2. Electrophoretic patterns of 1% solutions of the cancerous and noncancerous crude materials in 0.1 *M* veronal-HCl buffer (pH 8.6) containing 0.1 *M* NaCl (final ionic strength, 0.2). Temperature 15°C.

In Fig. 2 are shown the electrophoretic patterns of cancerous and noncancerous crude fractions examined in a Tiselius apparatus (Type HTD-1, Hitachi Ltd.). The noncancerous MPS III does not correspond to the cancerous MPS III with respect to electrophoretic mobility, although both of them were found to be the main components in the respective starting fractions. The noncancerous substance which electrophoretically corresponded to the cancerous MPS III may be contained in the Fr. III and IV in 2) c), but these fractions

were still not homogeneous and the further purification was unsuccessful. The content of the noncancerous MPS III appeared to be about one tenth of that of the cancerous.

Properties of MPSs III

Electrophoretic examination. The examination was carried out in a Tiselius apparatus (Type HTD–1, Hitachi Ltd.). Fig. 3 shows the electrophoretic patterns of 1% solution of MPSs III in an alkaline and an acidic buffer. Electrophoretic mobilities were measured at 15°C with 0.4% solutions in six buffers of various pH but of the same ionic strength 0.2. Either of the cancerous and noncancerous MPSs III showed apparently single boundaries in all these buffers. The results indicated that the canceorus MPS III was more acidic than the noncancerous.

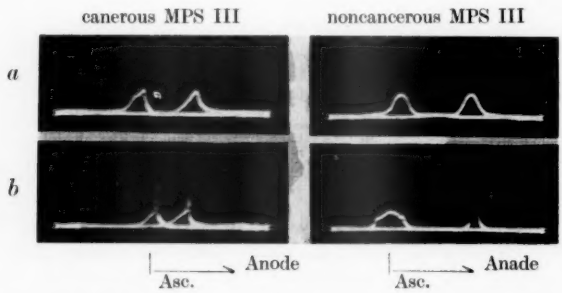


Fig. 3. Electrophoretic patterns of 1% solutions of the canceorus and noncancerous MPS III in 0.1 *M* veronal-HCl buffer (pH 8.6) containing 0.1 *M* NaCl (final ionic strength, 0.2) at 120 minutes run at 2.2 volts/cm (*a*) and of those in 0.2 *M* acetate buffer of pH 4.6 (I 0.2) at 50 minutes (canc. MPS III) or 120 minutes run (noncanc. MPS III) at 4.6 volts/cm (*b*). Temperature 15°C.

Mobilities in $cm^2 \cdot volt^{-1} \cdot sec^{-1} \times 10^{-5}$

pH	Buffer	Cancerous MPS III	Noncancerous MPS III
1.25	Glycine-HCl	0	0
1.4	"	0	0
2.0	"	−3.1	−0.6
4.6	Acetate	−5.9	−2.2
7.0	Phosphate	−6.8	−3.1
8.6	Veronal-HCl	−7.2	−4.9

Solubility and precipitability. The two substances behaved similarly in this respect. They are soluble in water at any pH value and in 90% (v/v) phenol. Insoluble in satd. $(NH_4)_2SO_4$ (room temperature), satd. Na_2SO_4 (33°C), glacial acetic acid, ethanol, acetone, and ether. They are not precipitated from aqueous

solutions with baryta, lead acetate, cupric chloride and trichloroacetic acid.

Test-tube tests. Both substances gave practically the same results in the following tests: Molisch ##, Neuberg-Saneyoshi −, biuret #, Hopkins-Cole (test for tryptophan)±, test for phosphorus −, test for hydrolyzable sulfur −. The cancerous MPS III gave strongly positive Bial and Dische reactions as well as direct and indirect Osaki-Turumi reaction, but the noncancerous gave faint Bial and Dische reactions and moderately positive direct and indirect Osaki-Turumi.

Paper-chromatography of constituents. Sugars. It was effected by the procedures similar to those described in the Second Report.[2] Both MPSs III were found to contain galactosamine, glucosamine, galactose and fucose as sugars, but no glucose nor mannose nor pentoses.

Amino acids. The substances were heated in sealed tubes with 6 *N* HCl in a boiling water bath for 24 hours. The occurring humin was filtered off and washed with water. The filterate and washing were freed from most of hydrochloric acid by evaporation *in vacuo* over anhydrous calcium chloride and solid caustic soda, being added with a few drops of water from time to time. The residues finally obtained were dissolved in a small amount of water and portions of solutions corresponding to 1 mg. substance before hydrolysis were sampled. An ascending two-dimensional chromatography of Masamune and Hakomori[7] with the solvents, butanol-acetic acid-water (4 : 1 : 2 by volume) and phenol–0.1 *M* borax (10 : 1 by volume), and a descending one-dimensional chromatography according to Högström[8] with the solvent mixture of methyl ethyl ketone-acetone-water (3 : 1 : 0.6 by volume) were applied. Toyo Roshi filter paper No. 50 was used and the spots were indicated with 0.1% ninhydrin in acetic acid-ethanol (3 : 7 by volume) mixture.

The chromatograms of the cancerous MPS III showed spots which corresponded to aspartic acid, glutamic acid, serine, glycine, threonine, alanine, tyrosine (faint), methionine, leucine and/or isoleucine, lysine, arginine, and proline.

Besides all these spots, two more spots were found which corresponded to valine and methionine in the chromatograms of the noncancerous MPS III.

Ultraviolet absorption. A photoelectric spectrophotometer (EPU-2A type, Hitachi Ltd.) was used and measurements were made in a cell of 1 cm. light path. The results are shown in Fig. 4. There was found an absorption maximum, though small, at 270 mμ in the spectrum of the noncancerous MPS III, whereas the maximum was scarcely found in that of the cancerous suggesting a difference in the content of aromatic amino acids.

Optical rotation. 0.01 *N* NaOH was the solvent. The noncancerous MPS III was found to be more levorotatory than the canceorus.

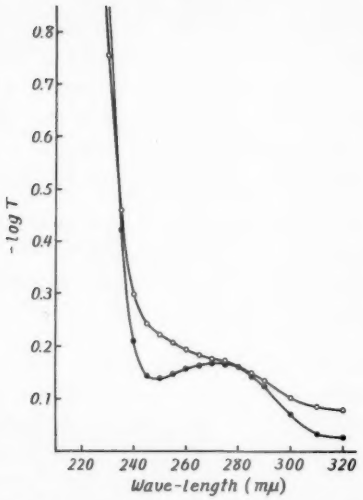


Fig. 4. Ultraviolet absorption spectra of 50 mg./dl. aqueous solutions of the cancerous (—o—o—o—) and noncancerous (—•—•—•—) MPS III.

Cancerous MPS III $[\alpha]_D^4 = \dfrac{-0.032 \times 1}{0.0110 \times 1} = -2.9°$

Noncancerous MPS III $[\alpha]_D^4 = \dfrac{-0.223 \times 1}{0.0094 \times 1} = -23.7°$

Quantitative composition. The analytical figures are embodied in Table I. There was no appreciable difference in total hexosamine contents between the cancerous and noncancerous MPSs III. Galactose and fucose contents are somewhat higher in the noncancerous, whereas the cancerous MPS III surpasses the noncancerous markedly in sialic acid contents.

Anemia-inducing potency (*KIK potency*). The potency was checked by the method of Masamune and Kawasaki.[12] As seen in Table II, both MPSs III were proved to be inactive.

Blood group potency. The glass plate method of Masamune *et al.*[13] was applied, substituting 1% suspensions of erythrocytes for 5% suspensions. The result is illustrated in Table III. The noncancerous MPS III showed a slight inhibitory activity to the agglutination of red cells, but the fact may most probably be due to the contamination with a faint amount of some highly active group mucopolysaccharides such as MPS I and IV.

Toxohormone activity. Only the cancerous MPS III was examined for the activity by the titrimetrical and volumetrical methods for catalase deter-

TABLE I. Per cent Compositions of MPSs III of Stomach Cancer and of Stomach Mucosa

Analysis	Stomach cancer	Stomach mucosa
N*	6.7	7.3
Total hexosamine†	26.4	25.0
Galactose‡	21.8	26.7
Fucose§	6.7	10.9
Sialic acid‖	18.3	1.4
Ash/	1.5	0

* Micro Kjeldahl. † Masamune and Yosizawa[9]. ‡ Reaction HR_{10} of Masamune and Sakamoto[6]. § A modification of Dische and Shettles.[10] ‖ Direct Ehrlich reaction of Werner and Odin.[11] / Pregl method without use of H_2SO_4.

TABLE II. Anemia-inducing Potency of MPSs III

MPS III	Dosis per kg. body wt. (mg.)	Rabbit Body wt. (kg.)	Sex	Number of red cells ($\times 10^4$, per mm^3) Before injection	Time after injection (hrs.) 1	2	3	4	5	Maximum decrease (%)
Cancerous	3	2.6	♂	637	661	643	658	657	647	
	〃	2.5	♂	514	**497**	526	536	534	501	3
	〃	3.2	♂	635	631	629	618	600	**596**	6
Noncancerous	1	2.5	♀	682	700	695	**674**	702	691	1
	〃	2.6	♂	650	659	667	672	**641**	649	1
	3	2.3	♂	583	592	586	609	577	**571**	2

TABLE III. Anti-hemagglutinative Potency of MPSs III

MPS III	Red cells	Serum	Dilution of the substances (1 : $10^3\times$) 1	2	4	8	16	32	64	128	256	512	∞
Cancerous	A	α	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	B	β	−	+	++	+++	+++	+++	+++	+++	+++	+++	+++
	O	Goat	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	O	Anti-E	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Noncancerous	A	α	−	−	+	++	+++	+++	+++	+++	+++	+++	+++
	B	β	−	−	+	+++	+++	+++	+++	+++	+++	+++	+++
	O	Goat	−	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	O	Anti-E	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Goat: a normal anti-O goat serum. Anti-E: an eel serum. − no hemagglutination, + faint hemaggl., ± slight hemaggl., ++ marked hemaggl., +++strong hemaggl. The sera were diluted to titre 16 before use.

mination.[14] The activity of the noncancerous MPS III was not determined becauses of shortage of the material. The results are shown in Table IV A–C. The cancerous MPS III acted as toxohormone when injected intraperitoneally into mice in dosage of 5 mg. *per capita*.

TABLE IVA. Liver-catalase Depression *in vivo* by Cancerous MPS III

No. of test-set	Per cent activity	
	With 5 mg. substance	With 1 mg. substance
1	80	77
2	72	76
3	69	77
4	70	94
5	71	
Average	72	81

Indicated amounts of substance were injected into mice intraperitoneally. The values in the Table represent the average of those obtained with three mice. Control experiments were performed also with three mice in all test-sets.

TABLE IVB. Effect of Cancerous MPS III on Kidney- and Blood-catalase *in vivo*

Mouse Number	Kidney	Blood	Liver
Oxygen evolved (ml.) in control experiments			
137	2.2	1.8	8.3
138	3.7	2.3	12.5
139	3.4	2.0	7.6
Average	3.1	2.0	9.5
Oxygen evolved (ml.) in test experiments			
140	2.6	2.3	6.2
141	2.9	2.3	7.1
142	3.5	2.2	6.8
Average	3.0	2.3	6.7
(% activity against the control)	(97)	(115)	(71)

5 mg. amounts of the substance were injected into mice. Liver-catalase activities were determined for comparison.

TABLE IVC. Effect of the Cancerous MPS III on Liver Catalase *in vitro*

Mouse Number	Liver-catalase activity (oxygen evolved in ml.)		
	Control I (No incubation)	Control II (Incubated without the substance)	Test (Incubated with the substance)
143	9·5	6.0	5.8
144	12.8	9.8	10.4
145	10.1	7.7	8.6
Average	10.8	7.8	8.6 (106% of Control II value)

The liver homogenates were incubated with 5 mg. of the substance (Masamune *et al.*[14]).

DISCUSSION

In the preceeding papers[2)-4)], we reported that sialic acid contents in mucopolysaccharides from gastric cancer tissues were higher than those correspondings from noncancerous. Our present study on MPSs III demonstrated the relation concerning sialic acid contents much remarkably. Also the observed big difference in sialic acid contents between cancerous and noncancerous MPSs III may account for the discrepancies in their electrophoretic patterns as well as the precipitabilities of these two comparable substances in barium acetate solution after the addition of ethanol.

SUMMARY

1. MPSs III, cancerous and noncancerous, were prepared by modified procedures of that described in the First Report[1)] and examined physically, chemically and biologically.

2. Electrophoretic mobility studies showed that the cancerous MPS III was more acidic than the noncancerous.

3. MPSs III were found to contain galactosamine, glucosamine, galactose, fucose and sialic acid as sugars. Total hexosamine contents were almost on the same level in both substances. The cancerous MPS III contained some what less amounts of galactose and fucose, but markedly larger amount of sialic acid than the noncancerous.

4. Either preparations do not possess group activity within ABO classification. They are also devoid of anemia-inducing activity. The cancerous MPS III was tested for toxohormone activity and found weakly active.

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References and Note

1) Masamune, Kawasaki, Abe, S., Oyama & Yamaguchi, Tohoku J. Exp. Med., 1958, **68**, 81.
2) Kawasaki, *ibid.*, 1958, **68**, 119.
3) Kawasaki, *ibid.*, 1959, **69**, 153.
4) Masamune, Kawasaki & Abe, S., *ibid.*, 1958, **68**, 173.
5) Type 426 Resin, the product of B. F. Goodrich Chemical Co., Cleveland 15, Ohio., U.S.A.
6) Masamune & Sakamoto, Tohoku J. Exp. Med., 1956, **63**, 345.
7) Masamune & Hakomori, *ibid.*, 1956, **64**, 59.
8) Högström, Acta chem. Scand., 1957, **11**, 743.
9) Masamune & Yosizawa, Tohoku J. Exp. Med., 1957, **65**, 169.
10) Masamune & Kawasaki, *ibid.*, 1956, **63**, 369 (ff. 381–382).
11) Werner & Odin, Acta soc. med. Upsal., 1952, **57**, 230.
12) Masamune & Kawasaki, Tohoku J. Exp. Med., 1956, **63**, 369 (f. 372).
13) Masamune, Hakomori, Numabe, Akama & Kamiyama, *ibid.*, 1955, **61**, 283 (ff. 294–295).
14) Masamune, Tsuiki, Kamiyama, Abe, S., Haga, Kawasaki, Kaketa & Abe, Sh., *ibid.*, 1958, **67**, 309.

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Studies on Experimental Influenza in Mice

IV. Growth Characteristics of the PR8 Virus in mouse Tissues Explanted in Vitro

By

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Growth characteristics of the PR8 virus in various organs of mice when the inoculum was given from three different routes have been already described[1]. Distribution of the inoculum virus labeled with P^{32} in these organs just after the introduction into the mice again from three different routes has been also described by pursuing the P count derived from the inoculum virus[2]. Taken together, the organs which allow the detectable virus growth as far as they are in vivo situation have been found to be lung, liver, spleen and kidney.

Growth of an influenza virus in organs other than lung was thought to be a matter of interest. Though the result described in the first paper[1] indicated the unusual growth characterisitics of the virus in the organs like liver, spleen and kidney, obtained evidence suggested at least the possible distribution of this virus through the body even after intranasal infection. This assumption has been further confirmed by detecting the radioactivity of blood, obtained from mice infected intranasally with P^{32} labeled virus.[2] Thus the distribution of the virus via blood stream is considered to be the case as far as experimental infection in mice is concerned. The fact described above may give an idea to consider murine influenza not as respiratory disease but rather as general systemic one. If this is so, encephalitic or cardiac symptoms found with human influenza cases[3] backed up with the evidence of detecting virus in these organs[4] on autopsy, will be quite conceivable because of the real distribution of the agent. Thus the pathogenesis of influenza infections should be reconsidered on the basis of these findings.

Having these considerations in one hand and also in an effort to find out suitable cell culture system which will allow us the detection of cytopathic effect of this virus on the other, growth of the virus in various mouse tissues has been tested in vitro.

To this end, three types of tissue cultures, i.e. Maitland, Zinsser's agar slant

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and roller tube cultures were prepared. The order described above was arranged in a fashion from a tissue culture without any cell growth to a tissue culture with full growth.

Facts have been presented that roller tube culture is most suitable at least in detecting the growth of virus in lung, liver and kidney tissues but not in spleen. However, any cytopathic changes have not been accompanied with the virus growth. On Zinsser's agar slant, accumulation of the virus was proved only with lung tissues.

MATERIALS and METHODS

Virus The PR8 strain of type A influenza virus was used. It has undergone seven passages in ferrets, 593 in mice and 170 in eggs. During these experiments, virus from 170th to 174th egg passage was used. Stock virus was obtained as harvested allantoic fluid of eggs after incubation at 37°C for 48 hrs, which had been infected with 10^{-4} dilution of the inoculum. Such fluid contained $10^{9.8}$ 50% egg infectious dosis (EID_{50}) and $10^{3.7}$ hemagglutinin (HA) per milliliter.

Assays The number of infectious particles was calculated by usual egg infectious dosis. Hemagglutinin titrations were made by Salk's pattern method. All assay specimens for hemagglutinin titration were pretreated with cholera filtrate by our standard procedure to remove non-specific inhibitors[5].

Tissue cultures Three types of cultures were examined in order to prove the specificity of each organs in allowing the growth of this virus. The first one is a classic Maitland type culture. Here 15-day-old chicken chorioallantoic membrane was served as the positive control and the virus released into the fluid was examined. Second one is the growth on Zinsser's agar slant. The amount of virus accumulated in the tissue fragment incubated on the slant was examined. Here the minced tissues of both chorioallantoic membrane and chick embryo obtained from 15-day-old egg were used as the control. Third type of culture employed was the usual roller tube culture.

Practical procedure in preparing tissue culture cells

1) Maitland type cultures

Brain, lung, liver, kidney, spleen, pancreas and muscle were removed aseptically from adult mice of white dd strain, the mice which had been used throughout this series experiment. After two times washings by changing Hanks' solution, they were cut into small pieces with the aid of scissors and 300 mg amount was distributed in each small test tube. 2 ml amount of Hanks' solution containing inoculum virus (finally 10^{-3} dilution of the stock virus) was then added to these tubes. The amount of virus in fluid was examined at 24 and 48 hrs after incubation at 37°C. Details to perform the experiment have been already

described elsewhere.[6]

2) Zinsser's agar slant cultures

Eight ml amount of Zinsser's agar medium (mixture of 4.5% purified agar, 2× Hanks solution and bovine serum in a ratio of 3 : 3 : 2) was solidified as a slant in test tubes. Firstly, 700 mg of tissue fragment, obtained as described above was infected in petri dishes with 0.3 ml of 10^{-2} diluted virus. They were incubated for 30 minutes at room temperature for the infection. Then the fragments were washed carefully with Hanks' solution in petri dishes to remove the inoculum. The tissues thus freed from the inoculum were collected with the aid of spatula and 200 mg amounts of tissues were put on each three slant. The remaining 100 mg was served as testing the inoculum virus titer. These tubes were incubated at 37°C and the virus assay was made at a day or 2 days interval as long as 4 or 5 days. In titrating the virus activity, these tissues obtained from 3 tubes were combined and ground in ball and mill to make a 10% suspension. Centrifuged supernatant at 3,000 rpm for 10 minutes was used for virus assays.

3) Roller tube cultures

Only in this experiment, tissues were obtained from suckling mice of 3-day-old dd strain, because of the reproducible cell growth. Brain, heart, lung, liver, kidney, spleen, pancreas and muscles were resected from mice aseptically and cut into a small piece of average 1 mm diameter. In each test tube, 6 pieces were fixed by means of plasma clot. 2 tubes were employed for one assay. YLE solution (0.5% lactalbumin hydrolysate and 0.1% yeast extract in Earle's solution with 0.45% glucose), bovine serum and chick embryo extract (1 : 1) were mixed up in a ratio of 65 : 30 : 5 and 2 ml amount was used as the growing medium in each tube. Though the growth rate was different from tissue to tissue, the best growth was usually obtained after 5 days incubation at 37°C. For the virus growth experiment, the maintenance solution which was composed of YLE, inactivated horse serum and CEE (1 : 1) with a ratio of 85 : 10 : 5 was used throughout. Then the inoculum was drawn off and these tissues were washed 3 times with 2 ml amount of Hanks' solution. Then the 2 ml amount of maintenance solution was refed to each and virus assays were made with these fluids.

EXPERIMENTAL

No detectable growth of the PR8 virus in Maitland type culture of various mouse organs

Practical technics to do this have been already fully described in a section of materials and methods. The results were illustrated by Fig. 1, where the virus titer in a fluid was examined after 24 and 48 hrs. of incubation. Chorioallantoic membrane served as the control gave a good growth of the virus

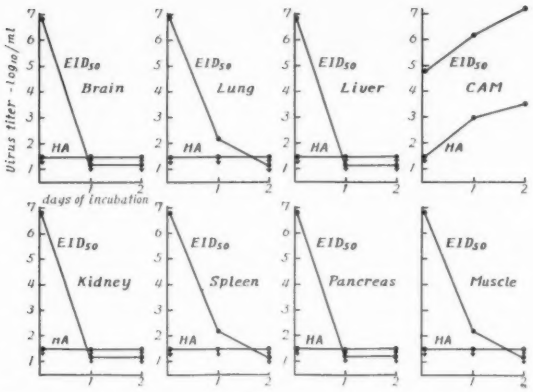

Fig. 1. Growth curves of PR8 virus in Maitland type cultures of various mouse tissues. Chorioallantoic membrane (CAM) was served as the control.

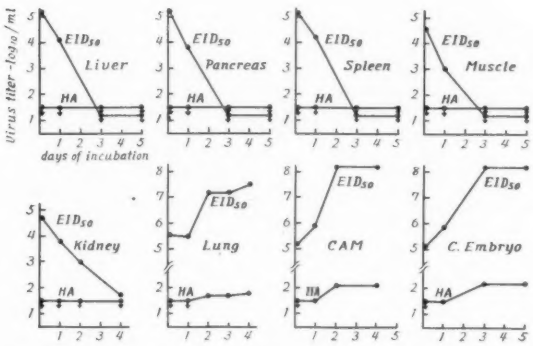

Fig. 2. Growth curves of PR8 virus on Zinsser's agar slant cultures of various mouse tissues. Chorioallantoic membrane (CAM) and chick embryo were served as controls. Amount of virus was examined by titrating both EID_{50} and HA with 10% homogenate of each.

when examined both by means of EID_{50} and HA. On the other hand, no measurable virus growth with mouse tissues such as brain, lung, liver, kidney, spleen, pancreas and muscle has been noticed. Only with tissues of the lung, spleen and muscle, there was a tendency to maintain the virus titer in a fluid a little better than with other tissues, when infectivity titration of the fluid was conducted with the harvests, obtained at 24 hrs. But the fact was not taken for

further considerations.

Virus growth in lung tissues on Zinsser's agar slant

In the second place, the accumulation of virus in tissues on Zinsser's agar slant was tested and the results were summarized in Fig. 2. The advantage of this culture when compared to the former Maitland type culture will be in the relative heat resistance of the virus inside the cell or attached to the cell when compared to the free virus. The result illustrated in Fig. 2 shows the growth of virus in lung tissues as well as in tissues derived from chick embryo and chicken chorioallantoic membrane. In this experiment, growth was pursued every day or every two days up to 4th or 5th day after infection, where the growth practically reached to the maximum after 2 or 3 days of incubation. Final growth in lung, however, was almost one tenth of that obtained in chick embryo or chicken chorioallantoic membrane. In lung tissues, HA was also detectable and I/A

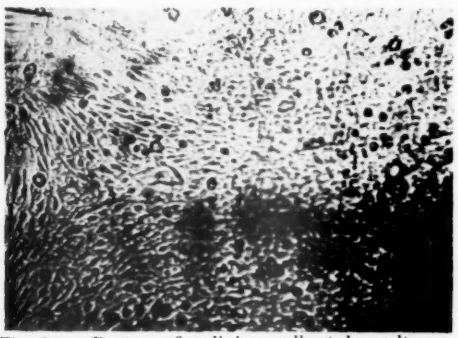

Fig. 3–a. Portion of a living roller tube culture of mouse lung tissues. ×80.

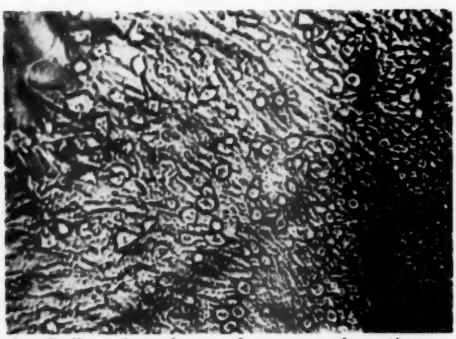

Fig. 3–b. Roller tube culture of mouse spleen tissues. ×80. Note the rounded or rather hexagonal cells extending outward.

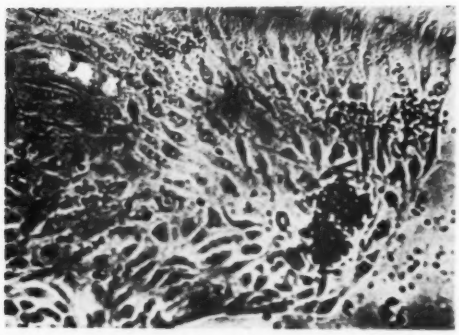

Fig. 3–c. Roller tube culture of mouse kidney tissues. ×80.

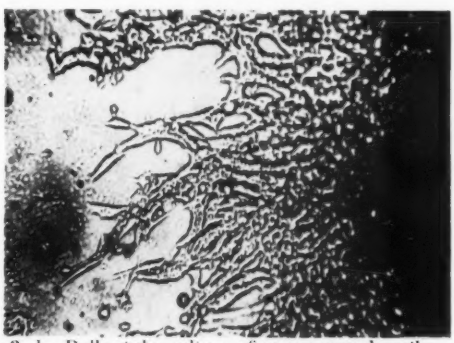

Fig. 3–d. Roller tube culture of mouse muscle cells. ×80.

(EID_{50}/HA) ratio was calculated as 5.7. Thus nearly complete growth was found in this condition.

Cell growth from various mouse organs in roller tube cultures

Except brain, all of the organs tested for the cell proliferation gave a good result (*see Fig.* 3).

Cell grown from lung, liver, heart and pancreas explants had predominantly the appearance of fibroblasts, but some other cells which had the appearance of epithelial cell or histiocyte were also detectable. In Fig. 3–a, maximum growth obtained with lung tissues was illustrated. Among the tissues tested so far, spleen gave the best growth. However, growth characteristics of the cell derived from spleen were different from the former four. Migration of round cells was first revealed around the explanted tissues. They were different from fibroblasts and some of them were rather hexagonal. Then an overgrowth of fibroblastic cells was followed and as a result the explant itself was digested. By these means,

almost monolayered fibroblast cultures with some scattered round cells of epithelial character were finally obtained (Fig. 3–b). With kidney, predominant epithelial cell growth occurred with some fibroblastic cell (Fig. 3–c). This feature was not different from that found with the same organ of the other animals. Colonial growth of epithelial cells were usually surrounded with the sheets of fibroblasts. With muscle (Fig. 3–d), only the growth of muscle cells were provable.

Growth of the PR8 virus in roller tube cultures

Tissues other than lung, kidney and liver did not allow the growth of the virus. These results were shown in Fig. 4, where the virus liberated into the liquid phase has been assayed at 48 hrs intervals. Brain tissues without appreciable cell growth was referred to serve as the control indicating the heat inactivation of the virus.

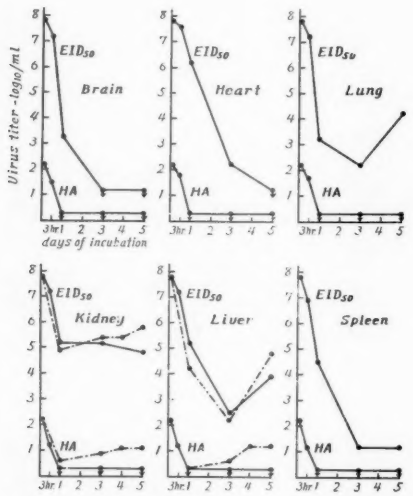

Fig. 4. Growth curves of PR8 virus in roller tube cultures of various mouse tissues. Differential virus assay was made every 2 days by titrating the EID_{50} and HA of culture fluid. Dotted line shows another experimental result, where accumulated titer was examined.

In kidney, growth tendency was not so discernible in such a differential response examination. Obvious eclipse period was not found as can be seen in the figure. However, the titer held relatively high up to the 5th day. Thus another experimental run was carried out where the titer in the fluid accumulated after appropreate incubation period was tested. In the figure, the dotted

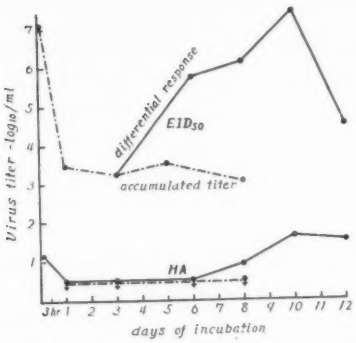

Fig. 5. Growth curves of PR8 virus in roller tube culture of mouse lung tissue. Two different system were adopted for testing virus growth (see text).

line showed the latter result and the growth tendency was now became evident. The shift of hemagglutinin titer was also detectable even if in low level.

In liver and lung, the same type of growth curve was obtained but the rise of infectious titer was first evident after 5 days of incubation. The latent period was thought to be rather longer than usual. Thus the accumulated virus titer was again exmained first with liver (dotted line in Fig. 4). In this case, the result was almost identical with that of differential response experiment. Thus the idea of slow maturation in these situation was hold. To approve this idea, again differential response experiment was repeated with lung tissues but for a longer period. This result has been illustrated by Fig. 5. In the figure, dotted line shows the accumulation curve where the significant rise of infectious titer is not obtainable. Contrary to this result, if differential response experiment was made for a longer period, the release of virus from the cell was shown to be fairly high but relatively slow. Exchange of medium seems to be favourable for virus liberation, at least in this condition.

With cultures derived from heart and spleen, no appreciable growth was recognized as shown in Fig. 4. This was also the case with cultures obtained from pancreas and muscle, thus the results were omitted.

Throughout the experiment, no cytopathic changes have been recognized with these tissues, as far as when they are examined microscopically without any staining. The fact together with the release of large amount of virus into the medium only when the medium was changed often as has been the case with lung tissues, may suggest the possibility of latent type infection but this was left for further studies.

DISCUSSIONS

It has been observed that a tissue entirely resistant to a viral infection in vivo may become highly sensitive to the same infection after explantation in vitro. This phenomenon is well illustrated by the behavior of the kidney of the monkey in which no multiplication of poliovirus occurs when the agent is inoculated directly in the organ in vivo[7]. However, monkey kidney cell supports a rapid multiplication of the same virus when separated from the organism and maintained in tissue culture. In turn, if the growth of virus was detectable in some organs in vivo, the fact may indicate that there may be a cell which will allow the growth of virus when they were maintained in vitro under proper condition. Having these considerations in mind, tissues derived from various mice organs were examined whether some of them which had been shown to allow the virus growth in vivo may support the growth of the same virus in vitro or not.

First of all, the lung tissue has been characterized by the fact that the reasonable amount of virus accumulation was shown even on Zinsser's agar slant. Moreover, the virus found in this condition was fairly complete in testing the I/A ratio.

Among the three types of cultures, here employed, roller tube culture was most suitable in detecting the growth of PR8 virus. Though the exact number of cells participated to the real virus multiplication was not determined because of the nature of cultures, cells derived from kidney and liver explants were also proved to allow the growth of virus in addition to that from lung. This fact should be taken into account in considering the pathogenesis of influenza infections in vivo, though does not approve the idea that the virus growth in these organs of mice is really the case.[1]

Attempts to propagate the virus in the culture derived from spleen has been failed in spite of the excellent growth of the cell mainly of fiborblastic appearance. Many explanations, such as the selected growth of some resistant cells in vitro, or influence of immune mechanisms or so on will be applicable but the fact was not pursued so far.

Unfortunately, cytopathic effect of the virus was not detectable in this experiment. The fact is concordant to many observations made on related subjects. However, in a light of Tyrrell's recent paper[8], the fact should be further examined in detail. Particularly the fluorescent antibody techniques will give us an indication in visualizing the kind of cell, really participating to the viral multiplication. This effort is now under progress and this kind of experiment may lead to a new conclusion in analyzing the necrotic effect of influenza virus on bronchial epithel, which has been believed to be the characteristic toxic action of the virus.

SUMMARY

The PR8 strain of influenza virus was examined on the growth ability in three types of tissue cultures derived from various mice organs.

In Maitland type culture, the release of virus into a medium was not detectable with tissues of brain, lung, liver, kidney, spleen, pancreas and muscle. Whereas the excellent proliferation was proved with chicken chorioallantoic membrane examined under the same condition. When these tissues were placed on Zinsser's agar slant, viral multiplication was found only with lung tissues. However, the amount of virus here found was almost one tenth of that obtained with chick embryo and chicken chorioallantoic membrane used as the control in the same way.

Roller tube cultures were carried out with explants derived from various mouse organs obtained from 3-day-old mice. Reasonable growth of the cell eligible for viral growth study was obtained with lung, liver, kidney, spleen, pancreas, heart and muscle. When they are infected in vitro, growth of virus was found in tissues of lung, liver and kidney in concordant with the result obtained in animal experiment. However, in tissues of spleen any growth signs were not obtained. Daily change of the medium was favourable in detecting the release of virus from the lung tissue into a medium.

References

1) Ishida, N., Mizuno, H. & Chiba, A., Tohoku J. Exper. Med., 1959, **71**, 137.
2) Ishida, N., Kosaka, Y. & Sasaki, Y., Tohoku J. Exper. Med., 1959, **71**, 151.
3) Silber, E.N., Ann. Int. Med., 1958, **48**, 228.
4) Kaji, M., Oseasohn, R., Jordan, W.S. & Dingle, J.H., Proc. Soc. Exp. Biol. & Med., 1959, **100**, 272.
5) Sasaki, H., Virus (Jap.), 1959, **9**, 404.
6) Ishida, N., Ansai, O. & Horigome, E., Virus (Jap.), 1958, **8**, 240.
7) Kaplan, A.S., Virology, 1955, **1**, 377.
8) Heath, R.B. & Tyrrell, D.A.J., Arch. ges. Virusforshg., 1959, **8**, 577.

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Anemia-inducing Substances from Stomach Cancer Tissue

I. On a Dialyzable Active Substance*

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Several KIK factors, anemia-inducing substances, which are found specifically in cancerous materials, were isolated previously in this Institute from cancerous gastric juice,[1] ascites[2)3)] and urines[4]. Chemical and biological properties of these substances were compared with those obtained from noncancerous corresponding sources by the same isolation procedures. All the substances thus obtained belonged to the class of Molisch-positive mucopolysaccharides or glucidamins in Masamune's terminology.

Previously, it was reported also that four kinds of KIK inactive mucopolysaccharide (MPS I-IV) were isolated after fractionation of water extracts of gastric cancer tissues.[5)6)] We tested the rest of the fractions thoroughly for the activity in KIK reaction and found three of them were active. The active fractions were subjected to further fractionation which resulted in the isolation of two anemia-inducing substances. The one from "Dialysates 1 and 2" in the previous paper was obtained in crystalline form and identified as *myo*-inositol. This product is not specific to cancer, because the same substance was obtained also from the noncancerous mucosa rather in higher yield. However, the other active substance which was isolated from the undialyzable fraction appeared to be a KIK factor, for the corresponding noncancerous substance was devoid of the anemia-inducing potency.

In this paper are presented the isolation and properties of the dialyzable active substance, *myo*-inositol, and in the accompanying paper will be reported the other, KIK factor, which belongs to the class of mucoproteins (glucidamins).

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* 232nd report of Masamune and coworker's "Biochemical Studies on Carbohydrates."

† Deceased in 1959.

EXPERIMENTAL

Preparation Procedure

Solid "Dialysates 1 and 2", which had been obtained as described[5] previously were used as starting materials. These were fractionated further as follows :–

1) Fractionation of cancerous dialysates

a) Cancerous dialysates (Dialysate 1 : Group A 4.3 g., B 2.6 g., O 1.6 g. ; Dialysate 2 : Group A 0.5 g., B 0.3 g., O 0.3g .) were combined, and 9.3 g. of the mixed substances were dissolved in 200 ml. of water. The solution (yellowish brown, slightly turbid, pH 6.8) was adjusted to pH 5.0 with 10% HCl. The resulting flocculent precipitate was centrifuged, washed twice with 40 ml. of water adjusted to pH 5.0 and with ethanol and then dried in a vacuum desiccator over anhydrous calcium chloride. A grayish brown powder weighing 420 mg. was obtained – **Ppt$_{pH5.0}$**.

The translucent supernatant and washings at pH 5 were put together and its pH was brought to 9.6 with 10% NaOH. The resulting flocculent precipitate was separated by centrifugation, washed and dried giving rise to 115 mg. of a white powder-**Ppt$_{pH9.6}$**.

The mother fluid and washing at pH 9.6 were combined and diluted with water to 500 ml. A yellowish brown, transparent solution was passed through columns (3.5 cm. in diameter, 26 cm. in length) of Amberlite IR 120 (H^+) and of Amberlite IR 45 (OH^-) in turn, at a flow rate of 10 ml. per minute. Each column was washed with 1.5 l. of water, at a flow rate of 15 ml. per minute. The final effluent (pH 6.6, water-clear) and washing were distilled together under reduced pressure to syrupy consistency, and the substances were precipitated with 150 ml. of ethanol, washed with ethanol and ether and then dried. A white powder weighing 300 mg. was yielded–**Fr. A**.

The column of Amberlite IR 120 was eluted with 2 l. of 1 *N* HCl and the eluate was neutralized to pH 5.6 with solid calcium hydroxide, concentrated *in vacuo* to a syrup, then subjected to precipitation by addition of ethanol. The precipitates were washed thoroughly with ethanol and dried *in vacuo* over anhydrous calcium chloride. 4.5 g. of a white powder were obtained – **Fr. B**.

The column of Amberlite IR 45 was eluted with 2l. of 0.3*N* NaOH, and the eluate was neutralized to pH 6.0 by shaking with Amberlite IR 120 (H^+). The resins employed were washed with 1 l. of water. The neutralized eluate and washing were combined, followed by further concentration, precipitation and so on similarly to above. A yellow hygroscopic powder weighing 3.5 g. was obtained – **Fr. C**.

b) Fr. A (290 mg.) was thoroughly kneaded with 5 ml. of 85% (v/v) phenol and centrifuged. The centrifugate was further treated six times with

85% phenol in a similar manner, washed with ethanol and ether and then dried. A white powder (196 mg.) was resulted–**Insolphenol**. Molisch faintly positive, biuret negative.

To the supernatants were added 5 volumes of ethanol and the precipitates were centrifuged, and dried after washing with three 30 ml. portions of ethanol and ether. 80 mg. of a white powder were obtained–**Solphenol**. Molisch strongly positive, biuret moderately positive.

c) **Insolphenol** (187 mg.) was taken up with 4 ml. of water and an insoluble part (trace) was filtered off by suction. To the clear filtrate were added 6 ml. of 99% ethanol drop by drop, then appeared crystals in long needles. After standing in a refrigerator for 6 hours, 4 ml. of ethanol were further added, stored overnight and filtered. The crystals collected were recrystallized from water by addition of ethanol. Yield 169 mg., when recrystallized by the same manner.

2) Fractionation of noncancerous dialysates

Noncancerous dialysates ("Dialysates 1" : Group A 5.1 g., B 5.7 g., O 5.6 g.; "Dialysate 2" : Group A 1.6 g., B 0.8 g., O 1.6 g.) were combined, and 14.0 g. of which were subjected to fractionation in the same way as with the cancerous. Yields of **PptpH5.0**, **PptpH9.6**, **Fr. A**, **Fr. B** and **Fr. C** were 0.42 g., 0.30 g., 0.547 g., 7.0 g. and 5.3 g., respectively. From 500 mg. of **Fr. A** were yielded 135 mg. of **Solphenol** and 355 mg. of **Insolphenol**. From 300 mg. of **Insolphenol** were obtained 271 mg. of the crystals with constant melting point at 218–220°C.

Properties and Identification of the Crystal

Qualitative test. Molisch negative, Scherer positive, optically inactive. Phosphorus, nitrogen, sulfur and halogen, all negative. When the crystals from the cancerous tissue were mixed with those from the noncancerous, the melting point was not depressed. Also, mixed melting point test with *myo*-inositol (Takeda Pure Chemicals Ltd., recrystallized from water-ethanol, m.p. 218–220°C) showed no depression. The infrared spectrum of the crystal was recorded in a Perkin-Elmer Model 21 recording infrared spectrophotometer with a NaCl prism, using KBr disk method. The spectrum obtained is shown in Fig. 1A, which coincides with that of *myo*-inositol (Fig. 1B).

Quantitative analyses. Decrease of weight did not occur when heated at 105°C *in vacuo* over P_2O_5 for 5 hours. No ash was contained.

Anal. Calcd. for $C_6H_{12}O_6$: C 40.00, H 6.71

Found C 39.86, H 6.79

The analyses described above confirm the identity of this product as *myo*-inositol.

Anemia-inducing potency. It was assayed as described by Masamune and Kawasaki[1], that is, various amounts of the preparations were dissolved in 1 ml.

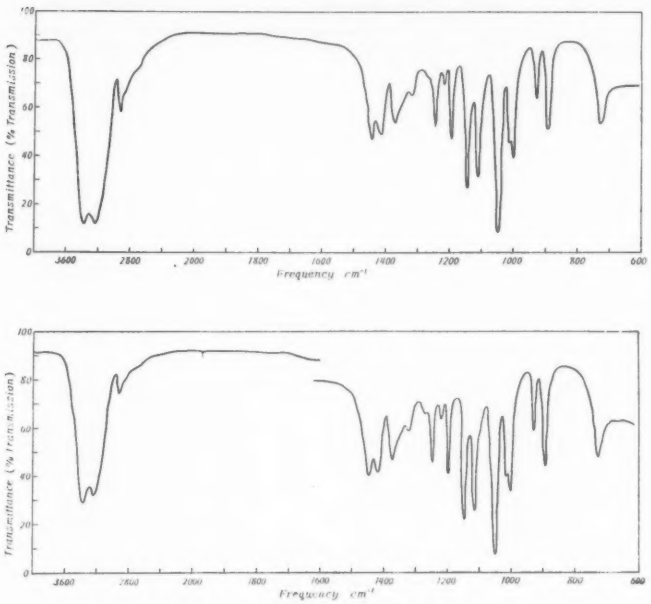

Fig. 1. Infrared spectra of the crystal from gastric cancer tissues (A) and authentic *myo*-inositol (B).

of physiological saline and injected into an auricular vein of rabbit, and numbers of red cells were counted before and hourly after the injection during 5 hours. As a considerable variation was expected to arise even when assayed with utmost care, we adapted the following criteria in judjing the activity of test substances: the minimum dosis of test substances per kg. body weight which caused in average 10% decrease in red cell numbers within a definite duration of time. In Table I are summarized the results with the final product as well as with intermediates from cancerous and noncancerous materials. In Table II is presented that of *myo*-inositol.

DISCUSSION

Haga[3] isolated from dialysates of cancerous ascitic fluids a KIK factor which differed to some extent from the undialyzable KIK factors KIK III_{1-2}[2] of the same source in quantitative-analytical figures and physical properties. This dialyzable factor, as well as the undialyzable ones, belonged to the class of mucoproteins (glucidamins) and gave positive Molisch and biuret reactions.

In order to determine whether a dialyzable active substance similar to Haga's

TABLE I. Effects of Fractions from Cancerous and Noncancerous Dialysates in Inducing Anemia in Rabbits

Dialysates	Fractions	Dosis per kg. body wt. (mg.)	Rabbit: Body wt. (kg.)	Rabbit: Sex	Number of red cells ($\times 10^4$, per cmm.): Before injection	Time after inj. (hrs.): 1	2	3	4	5	Maximum decrease (%)
Cancerous	Dialysate 1	1	2.7	♀	640	625	621	**563**	573	586	12
		〃	2.0	♂	662	652	617	582	**555**	596	16
	Dialysate 2	1	2.0	♀	586	578	562	**487**	498	508	17
		〃	3.3	♀	644	630	582	559	**511**	533	21
		〃	2.4	♂	632	633	602	538	**482**	536	24
	$Ppt_{pH5.0}$	1	3.0	♂	636	644	662	643	**631**	645	1
		〃	2.1	♂	634	646	684	670	**619**	630	2
	$Ppt_{pH9.6}$	1	2.3	♂	493	509	498	494	491	**485**	2
		〃	2.0	♀	632	630	623	641	**588**	601	7
	Fr. A	0.2	2.2	♂	515	519	512	462	**459**	496	11
		〃	2.0	♂	596	586	597	541	**524**	568	12
		〃	2.1	♂	555	575	514	512	**475**	504	14
		〃	2.1	♂	635	641	626	547	**522**	572	18
	Fr. B	2	2.6	♀	650	667	635	647	**632**	645	3
		〃	2.7	♂	653	645	649	**629**	**629**	645	4
		〃	2.5	♀	700	685	671	668	**650**	651	7
	Fr. C	1	2.3	♀	543	**535**	547	542	540	542	1
		〃	2.3	♂	583	590	595	**571**	585	591	2
		〃	3.3	♀	570	567	560	557	**550**	578	4
	Sol_{phenol}	0.5	2.3	♂	608	631	650	647	666	629	
		〃	2.2	♂	619	647	645	**610**	636	616	1
		〃	2.0	♀	509	525	530	511	**501**	519	2
		〃	2.0	♂	700	670	648	621	602	**591**	16
		1	2.5	♀	574	586	602	614	583	577	
		〃	2.0	♂	722	703	724	700	667	691	8
	$Insol_{phenol}$	0.2	2.1	♂	624	642	632	**553**	583	578	11
		〃	2.0	♂	694	680	657	619	**593**	635	15
		〃	2.3	♂	632	635	592	**529**	535	575	16
	Crystal	0.05	2.2	♂	572	569	571	580	542	**538**	6
		〃	2.1	♂	565	556	572	**517**	526	561	9
		0.1	2.4	♂	678	686	650	649	**622**	629	8
		〃	1.8	♂	535	503	502	492	**482**	555	10
		〃	1.8	♂	597	580	557	**523**	572	602	12
		〃	2.1	♂	572	527	506	**492**	532	560	14
		0.2	1.9	♀	547	565	537	511	**482**	545	12
		〃	2.3	♂	624	630	627	556	537	**532**	15
		〃	2.6	♂	638	631	621	**536**	584	580	16
		〃	1.7	♀	493	475	495	**380**	386	427	23
		0.5	2.0	♀	535	522	500	486	478	**440**	18
		〃	2.2	♂	555	563	551	487	**429**	449	23

TABLE I. Continued

Dialysates	Fractions	Dosis per kg. body wt. (mg.)	Rabbit Body wt. (kg.)	Sex	Number of red cells (×10⁴, per cmm.) Before injection	Time after inj. (hrs.) 1	2	3	4	5	Maximum decrease (%)
Noncancerous	Dialysate 1	1	2.3	♂	656	651	631	603	**581**	587	11
		〃	2.9	♀	665	651	598	**589**	599	678	12
	Dialysate 2	1	2.8	♂	705	692	618	604	**589**	618	14
		〃	2.1	♂	711	720	648	610	**562**	593	21
	Fr. A	0.5	2.5	♂	655	632	649	650	590	**574**	12
		〃	2.0	♂	649	642	623	592	571	**500**	23
		1	2.9	♂	594	596	603	576	544	**516**	13
		〃	2.6	♀	602	622	583	595	486	**456**	24
	Crystal	0.2	2.3	♂	643	626	618	593	**565**	567	12
		〃	2.3	♀	573	542	557	506	**501**	503	13
		〃	2.0	♂	753	748	755	636	**626**	724	17
		〃	2.6	♂	650	652	621	545	**534**	601	18
		0.5	2.2	♂	589	575	580	575	**517**	559	12
		〃	2.1	♂	655	673	708	566	**557**	597	15
		1	2.6	♀	723	732	695	644	**605**	613	16
		〃	3.1	♀	687	690	628	618	557	**499**	27

TABLE II. Anemia-inducing Potency of Commercial *Myo*-insoitol*

Dosis per kg. body wt. (mg.)	Rabbit Body wt. (kg.)	Sex	Number of red cells (×10⁴, per cmm.) Before injection	Time after inj. (hrs.) 1	2	3	4	5	Maximum decrease (%)
0.1	3.0	♀	652	660	681	637	**627**	643	4
〃	2.9	♀	561	544	569	532	537	**505**	10
〃	2.0	♂	751	753	721	714	718	**634**	16
0.2	2.2	♀	575	584	580	552	519	**500**	13
〃	2.1	♀	634	605	615	587	563	**540**	15
〃	2.6	♀	599	614	603	616	596	**500**	17
0.5	2.6	♀	671	716	696	646	**645**	673	4
〃	3.4	♂	668	689	674	648	**594**	616	11
〃	2.7	♀	470	456	458	465	**415**	424	12
〃	2.9	♀	471	467	457	435	**416**	440	12
〃	2.4	♂	547	565	515	499	**466**	504	15
〃	3.0	♂	612	615	581	562	546	**496**	19
〃	2.3	♀	577	566	442	460	**438**	498	24
1	2.5	♀	490	499	511	465	**434**	482	11
〃	2.0	♀	625	622	597	558	**517**	577	17
5	2.5	♀	703	682	**621**	624	636	661	12
〃	2.8	♀	610	582	570	565	**510**	561	16
50	2.4	♂	703	679	**614**	640	664	680	13
〃	2.3	♂	609	605	**499**	551	563	576	18

* From Takeda Pure Chemicals, Ltd., recrystallized from water-ethanol.

exists or not in the gastric cancer tissue, we treated the crude dialysate fractions from the cancer tissues by the similar procedure as used by Haga. Unexpectedly, however, the strongly Molisch-positive fraction, **Solphenol**, was found not to be active in inducing anemia and in contrast, the activity was found in the faintly Molisch-positive fraction, **Insolphenol**, from which crystalline *myo*-inositol was finally obtained as the active substance. Haga found in his dialysates no such fraction that would correspond to our **Insolphenol**[7]. It appears that the dialysate of the cancerous ascitic fluids contained no *myo*-inositol although it contained a KIK factor as an anemia-inducing substance. On the contrary, the dialysate of the water extract of gastric cancer tissues did not contain any KIK factor of mucopolysaccharide or mucoprotein nature.

Oh-Uti, Suzuki and Yamaguchi[8] extracted gastric cancer tissues with water, ethanol and physiological saline and fractionated the extracts. Based on the qualitative tests of the KIK-active crude fractions, they concluded that there were two anemia-inducing substances in gastric cancer tissues. The one of which was negative both in Molisch and biuret reactions and was dialyzable, suggesting its low molecular nature. The other was undialyzable and positive in the two test-tube tests, hence supposed to be a high molecular carbohydrate. This conclusion appears to agree well with our findings. However, they did not find the anemia-inducing activity in the dialysates of noncancerous gastric mucosae and their results indicated that the dialyzable active substance was soluble in ethanol. On account of these two fundamental inagreements with our present results, it is doubtful if their dialyzable active substance were *myo*-inositol. A possibility may not be neglected that there may be still other ethanol-soluble active substances in the dialysates.

Myo-inositol, one of the cyclohexitols, distributes widely in living bodies and has been considered to have some important biological activities, such as growth promoting activity for microorganisms, vitamin-like activities in higher animals, etc. (cf. Schopfer.[9])

Malangeau[10] reported that *myo*-inositols of either free and phospholipid forms are present in various tissues (muscle, kidney, liver) and in human blood plasma in which the values of free and lipidic *myo*-inositol were found to be 4–5 mg. and 3–3.5 mg. per liter respectively. Free *myo*-inositol is excreted daily in normal human urine in an average amount of 27 mg. per liter.[11] It is not clear as yet why the intravenous injection of such ordinarily occurring substance gives rise to anemia, though temporary, in rabbits. One must be cautious in using the KIK reaction for diagnostic purposes, for free *myo*-inositol is widely distributed in tissues and in body fluids.

SUMMARY

Two substances which have an anemia-inducing potency against rabbits

were isolated from gastric cancer tissues. One of the substances was dialyzable and identified as *myo*-inositol. The same substance was also obtained from noncancerous gastric mucosa rather in higher yield.

Acknowledgement

The authors are greatly indebted to Mr. H. Uda of Chemical Department for elementary analysis and to Mr. S. Aono of Chemical Research Institute of Non-aqueous Solutions for the determination of infrared spectrum. This work was aided by a grant for scientific researches from the Ministry of Education.

References and Note

1) Masamune & Kawasaki, Tohoku J. Exp. Med., 1956, **63**, 369.
2) Sugimoto, *ibid.*, 1959, **64**, 271.
3) Haga, *ibid.*, 1957, **67**, 43.
4) Kaketa, *ibid.*, 1958, **67**, 319.
5) Masamune, Kawasaki, Abe, S., Oyama & Yamaguchi, *ibid.*, 1958, **68**, 81.
6) Kawasaki, *ibid.*, 1958, **68**, 119; Masamune, Kawasaki & Abe, S., *ibid.*, 1958, **68**, 173; Kawasaki, *ibid.*, 1959, **69**, 153; Masamune, Kawasaki, Sinohara, Abe, Sh., & Abe, S., *ibid.*, 1960, **72**, 328.
7) Haga used 90% (v/v) phenol instead of 85%, but it does not seem to be a significant difference.
8) Oh-Uti, Suzuki & Yamaguchi, Tohoku J. Exp. Med., 1958, **67**, 115.
9) Schopfer, Bull. soc. chim. biol., 1951, **33**, 1113.
10) Malangeau, *ibid.*, 1956, **38**, 1003.
11) Courtois, Helleu, Malangeau & Perlès, *ibid.*, 1956, **38**, 1017.

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Anemia-inducing Substances from Stomach Cancer Tissue

II. A KIK Factor in Stomach Cancer Tissue*

By

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In the preceding paper[1] were reported the isolation and properties of an anemia-inducing substance, *myo*-inositol, from gastric cancer tissues and from gastric mucosae. This paper deals with another active substance, which is characteristic of cancer and belongs to mucoproteins.

EXPERIMENTAL

Preparation Procedure

Crude fractions, **Supern″$_{Ba(AcO)_2}$-60** prepared as described previously[2], were used as starting material. The fractions were found to have no blood group potency, so that all the fractions were combined and used as such.

1) Fractionation of cancerous material

a) The cancerous **Supern″$_{Ba(AcO)_2}$ 60** (430 mg.) was dissolved in a small volume of 0.1 *M* acetate buffer, pH 4.6, kneaded with Geon reson[3] (100–200 mesh) and the resulting paste was placed in a zone electrophoresis cell (34 × 7 × 1.5 cm.) in 1 cm. width, at 2 cm. distance from center to cathode side. Supporting medium used were the resin and buffer above described. After the zone electrophoresis in a refrigerator, the resin block was cut into 34 strips, each 1 cm. in width, and each of which was eluted with 20 ml. of water and assayed. The resulting electrophoresis pattern is shown in Fig. I. Eluates of fraction numbers 1–8, 9–12, 13–21 and 22–34 were combined respectively and named **Div. I, II, III** and **IV**. Each division was extracted twice with water, neutralized to pH 6 with sodium hydroxide, concentrated under reduced

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* 233rd report of Masamune and co-worker's "Biochemical Studies on Carbohydrates", 6th report on "Chemical Nature of KIK Factors".

† Deceased in 1959.

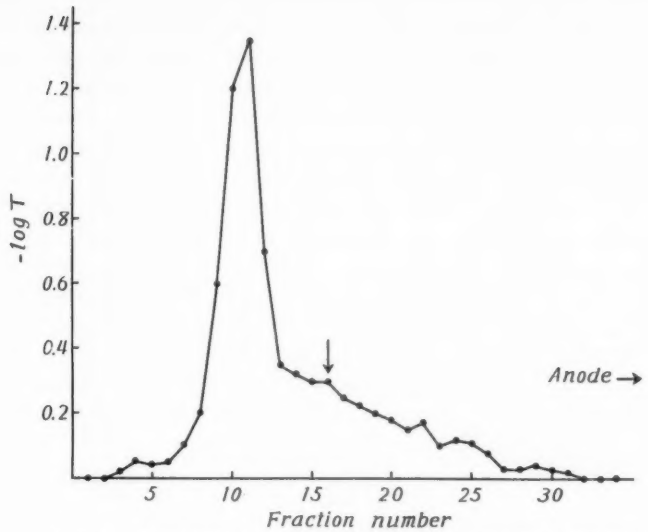

Fig. 1. Zone-electrophoresis pattern of the cancerous Supern $''_{Ba(AcO)_2\ 60}$. Cell (34×7×1.5 cm.) packed with Geon resin; buffer used, 0.1*M* acetate buffer pH 4.6; current 30 mA; duration 22 hrs. at 0±2°C. After electrophoresis, relative amount of hexoses in each fraction was expressed in terms of optical density after reaction HR_{10} of Masamune and Sakamoto.[4]

pressure to a syrup, precipitated by addition of ethanol which contained sodium acetate. The sediment was washed with ethanol and ether and then dried in a vacuum desiccator over anhydrous calcium chloride. Yields of Div. I, II, III and IV were 20 mg., 200 mg., 120 mg. and 50 mg., respectively.

Several batches of the material were treated same way as above and corresponding divisions were put together.

b) Div. II (500 mg.) was dissolved in 25 ml. of 90% (v/v) aqueous phenol. To the solution (transparent, yellowish brown) was added fractionally ethanol which contained 1% sodium acetate. The precipitates at ethanol concentration of 20, 40, 60 and 90% were centrifuged separately, and dried after washing twice with ethanol and once with ether.

Yields as follows:

Ppt. at ethanol concn. range of	0–20%	**(Ppt′Phenol 0~20)**	20 mg.
〃	20–40%	**(Ppt′Phenol 20~40)**	93 mg.
〃	40–60%	**(Ppt′Phenol 40~60)**	124 mg.
〃	60–90%	**(Ppt′Phenol 60~90)**	250 mg.

c) **Ppt′Phenol 60~90** (200 mg.) was dissolved in 5 ml. of water. To the

yellow clear solution was added an equal volume of 10% trichloroacetic acid under cooling on cracked ice. The resulting flocculent precipitate was separated by centrifugation, washed twice with 30 ml. of ethanol and once with ether and dried to yield 60 mg. of a yellowish gray powder — **Ppttca.**

The supernatant (transparent, faintly yellowish brown) was diluted with water to 100 ml., and neutralized by shaking with about 100 ml. (wet) of Amberlite IR 45 (OH^-) and then filtered. The resin was washed three 100 ml. portions of water. The filtrate and washings were combined, distilled under reduced pressure to a syrup, precipitated by addition of ethanol, washed twice with ethanol and once with ether and then dried in a vacuum desiccator. A yellowish white powder weighing 120 mg. (0.011% of the starting dry tissue material) was obtained — **cancerous MPR (Mucoprotein) I** (KIK factor).

2) Noncancerous **Supern″$_{Ba(AcO)_2-60}$** was subjected to the same fractionation. From 2.7 g. of **Supern″$_{Ba(AcO)_2-60}$**, yields of Div. II, **Ppt′$_{Phenol-60\sim90}$** and noncancerous MPR I (a substance which should correspond to the KIK factor) were 1.4 g., 650 mg. and 220 mg., respectively.

The yield of the noncancerous MPR I was 0.004% of the starting dry tissue material.

Properties of the KIK Factor and Corresponding Noncancerous Substance

Anemia-inducing potency. The potency was measured as described previously[1]. The results with the final product, some intermediates and starting material are shown in Table I. Marked anemia was induced in rabbits by intravenous injection of 0.1 mg. cancerous MPR I (KIK factor) per kg. of body weight, whereas the injection of noncancerous MPR I as much as 1 mg. per kg. body weight did not induced anemia.

Table I. Anemia-inducing Potency of Cancerous and Noncancerous Fractions

Source	Fractions	Dosis per kg. body wt. (mg.)	Rabbit Body wt. (kg.)	Rabbit Sex	Number of red cells ($\times 10^4$, per mm^3) Before injection	Time after injection (hrs.) 1	2	3	4	5	Maximum decrease (%)
Stomach cancer	Supern″$_{Ba(AcO)_2-60}$ (starting material)	0.3	2.3	♂	543	537	548	498	**487**	522	10
		〃	2.4	♂	585	586	598	536	**499**	535	15
		1	2.2	♀	537	526	538	534	**436**	450	19
		〃	3.0	♂	666	644	596	585	541	**529**	21
		〃	2.5	♀	656	655	666	622	**504**	530	23
	Div. IV	0.5	3.1	♂	724	736	754	718	**702**	731	3
		〃	2.3	♂	683	687	**650**	676	655	692	5
		〃	2.8	♂	628	603	**593**	615	608	600	6

TABLE I. Continued

Source	Fractions	Dosis per kg. body wt. (mg.)	Rabbit Body wt. (kg.)	Sex	Number of red cells ($\times 10^4$, per mm^3) Before injection	Time after injection (hrs.) 1	2	3	4	5	Maximum decrease (%)
Stomach cancer	$Ppt'_{phenol\ 20\sim40}$	0.2	3.0	♀	643	656	663	658	**607**	625	6
		〃	3.0	♀	598	599	**559**	620	566	561	7
		0.5	2.9	♂	597	614	574	597	604	**570**	5
		〃	2.7	♂	719	712	687	691	681	**680**	5
	Ppt_{tca}	0.5	2.5	♂	523	555	526	526	517	**513**	2
		〃	2.6	♀	650	667	635	**632**	645	653	3
		〃	2.5	♂	582	591	573	592	550	**549**	6
	MPR I (KIK Factor)	0.03	2.4	♀	652	672	636	**615**	632	638	6
		〃	2.3	♀	760	755	730	706	**696**	730	8
		〃	2.4	♀	553	543	540	527	506	**475**	14
		0.1	2.8	♀	575	579	584	**508**	509	535	12
		〃	2.3	♀	650	649	632	602	**561**	565	14
		〃	2.4	♀	714	695	688	626	**566**	629	21
		0.2	2.4	♂	571	589	546	545	**501**	519	12
		〃	2.6	♂	710	699	655	636	609	**594**	16
		〃	2.7	♀	533	526	444	**437**	441	456	18
		〃	3.2	♀	605	600	573	574	**481**	537	21
Stomach mucosa	$Supern''_{Ba(AcO)_2\ 60}$ (Starting material)	0.5	2.1	♀	658	675	689	653	655	**626**	5
		〃	2.3	♂	645	654	634	640	**511**	625	5
		1	2.8	♀	505	531	**495**	512	534	502	2
		〃	3.0	♂	623	618	615	611	**603**	607	3
		〃	2.4	♂	619	627	648	644	609	**596**	4
	MPR I	0.5	2.0	♂	574	604	579	556	**551**	552	4
		〃	2.4	♀	571	565	546	547	552	**542**	5
		〃	2.0	♀	652	652	626	627	**621**	625	5
		〃	2.1	♀	622	637	547	527	**517**	530	17
		1	3.0	♂	710	717	693	694	**677**	687	5
		〃	2.6	♂	644	666	624	615	**598**	607	7
		〃	2.6	♂	645	657	636	620	**594**	617	8

TABLE II. Liver-catalase Depression *in vivo* by KIK Factor and Corresponding Noncancerous

Indicated amounts of substance were injected into mice intraperitoneally. The values in the Table represent the average of those obtained with three mice. Control experiments were performed also with three mice in all test-sets.

No. of test-set	Per cent activity			
	With KIK Factor		With noncanc. MPR I	
	5 mg.	1 mg.	5 mg.	1 mg.
1	89	96		
2	84	100		
3	81	86		
4			84	88
5			109	100

Toxohormone activity. The activity was determined according to the method of Masamune and others[5]. As shown in Table II, the injection of 5 mg. of MPRs I did not cause depression of liver catalase in mice, that is, MPRs I were shown to be inactive as toxohormone.

Blood group potency. The glass plate method of Masamune and others[6] was applied, substituting 1% suspensions of erythrocytes for 5% suspensions. MPRs I were not active at all.

Electrophoresis. Examined in a Tiselius apparatus (HTD-1 type, Hitachi Ltd.). As shown in Fig. 2, either substances were proved to be not completely homogeneous. They also showed reversible boundary spreading, but further purification was not attempted because of their poor yields.

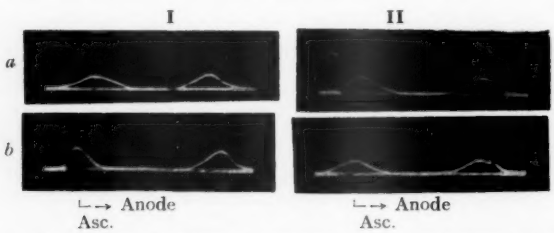

Fig. 2. Electrophoretic patterns of 1% solutions of the cancerous (*a*) and noncancerous (*b*) MPR I in 0.1 *M* veronal-HCl buffer (pH 8.6) containing 0.1 *M* NaCl (final ionic strength 0.2) after 60 minutes at 2.2 volts/cm (I) and of those in 0.2 *M* acetate buffer (pH 4.6) (I 0.2) after 60 minutes at 4.6 volts/cm (II). Temperature 15°C.

Mobility in $cm^2 . volt^{-1} . sec^{-1} \times 10^{-5}$

Canc. MPR I (KIK Factor)	in veronal-HCl buffer	−4.0
	in acetate buffer	−1.2
Noncanc. MPR I	in veronal-HCl buffer	−3.2
	in acetate buffer	−1.0

Solubility and precipitability. Both substances behaved similarly in this respect. They are soluble in water (pH 1~10), 90% (v/v) aqueous phenol and glacial acetic acid. Insoluble in saturated ammonium sulfate (at room temperature), saturated sodium sulfate (at 33°C), ethanol, acetone and ether. From aqueous solution, they are not precipitated with baryta, cupric chloride, lead acetate and trichloroacetic acid.

Test tube tests. Both substances gave the following results: Molisch ±, direct and indirect Osaki-Turumi +, Bial +, Dische −, biuret ±, Hopkins-Cole (test for tryptophan) +, tests for phosphorus and hydrolyzable sulfur −.

Paper chromatography of the components. 1) Sugars. 18 mg. of the substance were hydrolyzed with 2 ml. of 1 *N* H_2SO_4 by heating in a sealed tube at 100°C for 5 hours. The hydrolysate was neutralized with saturated baryta

to pH 6.6. The resulting precipitate was separated by centrifugation and washed with three 2 ml. portions of water. The supernatant and washings were passed through a column (1×5 cm.) of Amberlite IR 120 (H^+) and the column was washed with 30 ml. of water. The effluent was evaporated to dryness in a desiccator over anhydrous calcium chloride. The residue was dissolved in 0.1 ml. of water, spotted on two paper strips (Toyo Roshi No. 50) and developed by descending chromatography, the one with *n*-butanol-pyridine-water (5 : 3 : 2 v/v)[7] and the other with *n*-butyl acetate-acetic acid-ethanol-water (3 : 2 : 1 : 1 v/v)[7], at 24±1°C for 26 hours. Sugar spots were located by spraying 2% aniline hydrogen phthalate in water-saturated butanol. Either substances, cancerous and noncancerous, were found to contain galactose, glucose, mannose and fucose. No spots were indicated at the positions corresponding to pentoses.

The column of Amberlite IR 120 was then eluted with 50 ml. of 0.5 *N* HCl. The eluate was distilled under reduced pressure to dryness. The still-residue was dissolved in 20 ml. of water, and neutralized with Amberlite IR 45 (OH^-) to pH 6.0, filtered and washed with 50 ml. of water. The filtrate and the washing were combined, condensed under reduced pressure to dryness. The residue was dissolved in 0.1 ml. of water, and 0.05 ml. of the solution was subjected to descending paper chromatography. Solvent mixture, *n*-butanol-pyridine-0.25 *N* HCl (cf. Masamune and Yosizawa[8]); temperature, 24±1°C; duration, 40 hours; indicator, aniline hydrogen phthalate. Either substances were found to contain glucosamine and galactosamine.

2) Amino acids. Each substance was heated with 6 *N* HCl in a sealed tube for 24 hours at 100°C. The hydrolysate was evaporated to dryness at 30°C over anhydrous calcium chloride and solid caustic soda. The residue was dissolved in a few drops of water, then evaporated. This procedure was repeated several times until most of the hydrochloric acid was expelled. The residue finally obtained was dissolved in water, and the solution (equal to 0.75 mg. of the original substance) was subjected to ascending two-dimensional paper chromatography by the method of Masamune and Hakomori[9] and descending one-dimensional chromatography by the method of Högström[10] using methyl ethyl ketone-acetone-water (3 : 1 : 0.6 v/v) as the solvent. Toyo Roshi filter paper No. 50 was used. The indicator was 0.1% ninhydrin in acetic acid-ethanol (3 : 7 v/v) mixture. Cancerous MPR I (KIK factor) was found to contain aspartic acid, glutamic acid, serine, glycine, threonine, alanine, tyrosiine, valine, methionine, phenylalanine, leucine and/or isoleucine, proline, arginine and lysine. Noncancerous MPR I was devoid of methionine, but contained all other amino acids as found in the cancerous. Quantitative analysis of amino acids in these two substances are shown in Table III.

Ultraviolet absorption. A photoelectric spectrophotometer (EPB-U type, Hitachi Ltd.) was used. Measurements were made in a cuvette of 1 cm. light

path. As shown in Fig. 3, both substances gave spectra with absorption maximum at 275 mμ.

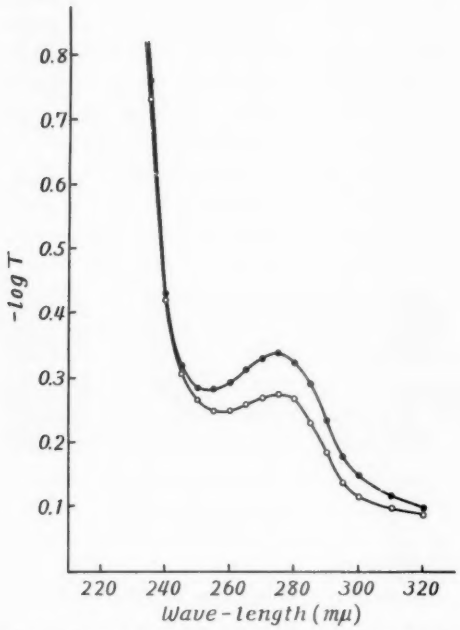

Fig. 3. Ultraviolet absorption spectra of 40 mg% aqueous solutions (pH 6.6) of the cancerous (—○—○—○—) and noncancerous (—●—●—●—) MPR I.

Optical rotation. Examinations were made with their aqueous solutions (pH 6.6). The cancerous MPR I was more levorotatory than the noncancerous.

Cancerous MPR I (KIK factor)

$$[\alpha]_D^5 = \frac{-1.045 \times 1}{0.0100 \times 1} = -104.5°$$

Noncancerous MPR I

$$[\alpha]_D^5 = \frac{-0.742 \times 1}{0.0100 \times 1} = -74.2°$$

Quantitative analyses. The analytical figures obtained are embodied in Table III. With respect to the contents of amino acids as well as sugars, there is no marked difference between the cancerous and noncancerous substances.

Table III. Composition in per cent of MPRs I

Analysis	Cancerous MPR I (KIK Factor)	Noncancerous MPR I
N*	12.1	11.2
Total hexosamine†	4.9	4.1
Hexoses as galactose‡	6.4	5.1
Fucose§	0.9	1.0
Sialic acid‖	2.0	1.5
Ash#	1.6	4.4
Amino acids**		
aspartic acid	13.4	14.1
glutamic acid	14.5	16.6
serine	6.4	7.5
threonine	4.8	4.7
glycine	12.0	10.0
alanine	7.1	6.0
tyrosine	0.3	0.2
valine	2.2	1.7
methionine		—
phenylalanine, leucine and/or isoleucine	4.5	4.5
proline	12.5	13.2
arginine and lysine	7.1	4.4

* Micro Kjeldahl. † Masamune and Yosizawa[8]. ‡ Although galactose, glucose and mannose were detected paperchromatographically, they were not determined separately but as galactose with reaction HR_{10} of Masamune and Sakamoto.[4] § A modification of Dische and Shettles.[11] ‖ Direct Ehrlich reaction of Werner and Odin.[12] # Pregl method without use of conc. H_2SO_4. ** Masamune and Hakomori[9] (Solvent phenol-0.1 *M* borax (10 : 1) was used instead of the solvent phenol-0.1 *M* borax (86 : 14)).

DISCUSSION

KIK factors had been prepared from cancerous gastric juice[13], ascites[5][14][15] and urines[16] in this Institute. As the control experiments, in each case, corresponding noncancerous materials, e.g., noncancerous gastric juice, ascites of patients suffering from liver cirrhosis or tuberculosis, and normal urines, had been fractionated with the same procedures that were applied to isolate respective KIK factors. Noncancerous or normal substances obtained in this way did not possess the anemia-inducing activity, although their chemical characteristics were very similar to that of respective KIK factor. The starting "cancerous" materials which had been used by previous workers contained obviously a big amount of "noncancerous" or normal materials. Therefore, KIK factor preparations obtained previously should be taken as mixtures of "essential" KIK factor (which is supposed to be strictly cancerous) and other noncancerous corresponding substances. This was formerly discussed by Masamune and Kawasaki[13] on the case of gastric juice.

In this study, we took gastric cancer tissues and noncancerous gastric mucosae as one of the most suitable material, for such purpose of separating "essential" KIK factor(s) and of ascertaining whether the noncancerous corresponding substance(s) would really exist or not. As described in the preceding section, we could isolate an "essential" KIK factor from gastric cancer tissues and inactive corresponding substance from gastric mucosae. Chemical analysis and other examinations of these two substances revealed that they are very similar. Both substances gave practically the same electrophoretic pattern, but found to be not completely homogeneous. Further purification was not attempted because of their poor yields. Therefore, it is not clear as yet if noncancerous tissues involve really an inactive corresponding substance, in spite of chemical and physicochemical similarities of these isolated substances. We are inclined, however, to assume that the existence of an inactive corresponding substance in normal tissues is highly probable. If we admit this possibility, the anemia-inducing activity of KIK factor(s) may be due to partial structural deviation from the corresponding noncancerous.

In this connection, it is of interest to note Kaketa's idea that KIK factors in secretions and excretions are formed by the cells normally secreting the corresponding non-anemia-inducing substances which differ from source to source and the transformation of non-anemia-inducing substances to KIK factors in such cells is supposed to be effected by some factor(s) from cancer tissues. However, there has not been any direct proof for the disorder of normal cells and for the mechanism by which cancer leads to the disorder in the distant normal cells.

SUMMARY

A KIK factor was isolated from gastric cancer tissues and compared with the corresponding inactive substance which was obtained from noncancerous gastric mucosae. Both substances belonged to the class of glucidamins in Masamune's terminology.

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References and Note

1) Masamune, Kawasaki & Sinohara, Tohoku J. Exp. Med., 1960, **72**, 348.
2) Masamune, Kawasaki, Abe, S., Oyama & Yamaguchi, *ibid.*, 1958, **68**, 81.
3) Type 426 Resin, the product of B.F. Goodrich Chemical Co., Cleveland 15, Ohio., U.S.A.
4) Masamune & Sakamoto, Tohoku J. Exp. Med., 1956, **63**, 345.
5) Masamune, Tsuiki, Kamiyama, Abe, S., Haga, Kawasaki, Kaketa & Abe, Sh., *ibid.*, 1958, **67**, 309.
6) Masamune, Hakomori, Numabe, Akama & Kamiyama, *ibid.*, 1955, **61**, 283 (ff. 294–295).

7) Masamune & Yosizawa, *ibid.*, 1953, **59**, 1.
8) Masamune & Yosizawa, *ibid.*, 1957, **65**, 169.
9) Masamune & Hakomori, *ibid.*, 1956, **64**, 59.
10) Högström, Acta chem. Scand., 1957, **11**, 743.
11) Masamune & Kawasaki, Tohoku J. Exp. Med., 1956, **63**, 369 (ff. 381–382).
12) Werner & Odin, Acta soc. med. Upsal., 1952, **57**, 230.
13) Masamune & Kawasaki, Tohoku J. Exp. Med., 1956, **63**, 369.
14) Sugimoto, *ibid.*, 1956, **64**, 271.
15) Haga, *ibid.*, 1957, **67**, 43.
16) Kaketa, *ibid.*, 1958, **67**, 319.

Tohoku J. Exper. Med., 1960, 72, 366-376

Paroxysmal Discharges of Cat's Cerebral Cortex treated with Gamma Aminobutyric Acid

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INTRODUCTION

The effects of gamma aminobutyric acid (GABA) upon electrocortical activity have been studied by a number of workers[1-5] with anesthetized or unanesthetized animals by applying the substance topically to the exposed cortical surface. A consistent finding has been obtained that when GABA acts upon the primary sensory evoked potential consisting of a positive wave followed immediately by a negative wave of 10–30 msec in duration, there occurs a rapid reversible abolition of the negative wave, with the positive wave unaffected or slightly increased in amplitude. Furthermore, the spontaneous spindling or the evoked recruiting response, both consisting of sharp, predominantly negative waves, has been found to turn into a train of positive waves under the action of GABA. Thus it seems well-established that the principal effect of this chemical substance upon electrocortical activity is a selective depressant action upon the sharp negative wave of 10–30 msec in duration, although Yamamoto and his co-workers[6] reported that the substance, when used in a high concentration, exerted a suppressing action upon the positive wave as well.

The experiment to be reported here is concerned with an effect of GABA which is seemingly distinguishable from the suppression of the sharp negative wave. We found that when animals were put under an appropriate depth of anesthesia, GABA-treated cortical points gave rise to high-voltage, slow potential changes which can properly be called paroxysmal discharges. The purpose of the present paper is to report some of the properties of this cortical paroxysmal activity.

EXPERIMENTAL

Method

Experiments were performed with cats weighing 1.5 to 3 kg. Surgical operations such as tracheal cannulation and exposure of the cerebral cortex were done mostly under ether anesthesia. In some cases pentobarbital sodium or

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thiopental sodium was used instead of ether, keeping the dose as small as possible.

Electrical activity was led off from the exposed cortical surface with a ball-tipped silver electrode, putting an indifferent electrode on inactive tissues. Amplification and registration of the electrical activities were made with the aid of a San'ei ink-writing electroencephalograph, the time constant of which was set at 0.5 or 0.3 seconds.

GABA, for the use of topical application to the exposed cortical surface, was made 0.1 to 1.0% in concentration in a physiological saline solution.

Results

Throughout the present experiment it was unfailingly confirmed that upon topical application of GABA waves of the spontaneous spindling changed their polarity from negative to positive (phase-inverted spindling). In addition, there occurred very often spontaneous paroxysmal discharges which were most marked during lulls of the spontaneous spindling and were sustained indefinitely as long as GABA remained on the cortical surface. The amplitude of the paroxysmal discharges was as high as several hundred microvolts under most favorable conditions.

1. *Morphology of the GABA-induced paroxysmal discharge*

Three typical patterns of the GABA-induced paroxysmal activity are reproduced in Fig. 1. They are distinguished from each other by the dominant component constituting the potential wave. Pattern A is characterized by monophasic positive spikes which occur regularly at a frequency of about 3 cps but sometimes appear sporadically. In pattern B there are sharp positive spikes occurring at a frequency of about 3 cps, and most of them are followed by large, slow waves. In contrast with these patterns, pattern C has no conspicuous spiky deflections, being composed of a train of regular slow waves. The frequency

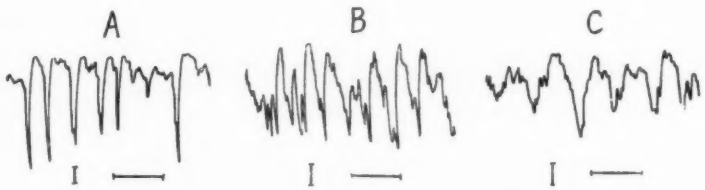

Fig. 1. Selected EEG tracings of GABA-induced paroxysmal activity. In this and subsequent figures, negativity of pick-up electrode on the cortical surface is indicated by upward deflection. Time, 1 second. Voltage scale, 200 microvolts.

of the slow wave in this case is rather low, covering the range from 0.5 to 3 cps. Thus it seems likely that the GABA-induced paroxysmal discharge has two components; one of them is the positive spike and the other the slow wave. Though several attempts have been made to reveal differences in property between the two components, no clear-cut results have been obtained. In the following, therefore, we shall deal with the paroxysmal activity without making any distinction between the spiky and the wavy configuration.

2. *Depth of anesthesia as a factor for maintaining the GABA-induced paroxysmal discharge*

Whether or not topical application of GABA to the exposed cortical surface gives rise to the paroxysmal activity depended largely upon the depth of anesthesia of the animal. First, too deep anesthesia was found to prevent the paroxysmal discharges from appearing with high amplitudes. This is shown in Fig. 2. Before applying GABA, the animal was put under a very light anesthesia produced by thiopental sodium (Fig. 2, A). At this time the cortex showed the spontaneous spindlings with sharp negative waves of high amplitude and also low-voltage, fast activity which prevailed during lulls of the spontaneous spindling. Under this condition of cortical activity, slow waves of high amplitude, occasionally associated with positive spikes, appeared in response to topical

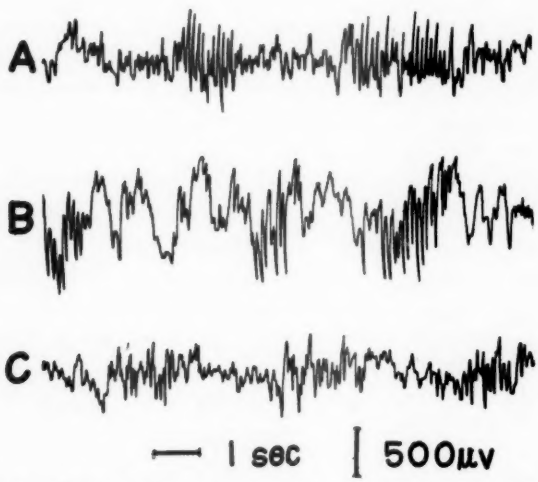

Fig. 2. Effect of deepening anesthesia. A & B, before and 3 minutes after application of GABA. Anesthesia was very light. Following GABA-treatment, paroxysmal activity appeared and spontaneous spindlings were reversed in phase. C, 1.5 minutes after administrating a small amount of pentobarbital sodium. Paroxysmal activity was markedly reduced.

application of GABA, which was also effective in reversing the phase of the spontaneous spindling (Fig. 2, B). Then, a small amount of pentobarbital sodium was given to the animal intravenously. The result of deepening the anesthesia was such that the paroxysmal slow waves were markedly reduced in amplitude with a slight suppression of the phase-inverted spontaneous spindlings (Fig. 2, C).

On the other hand, arousal of the lightly anesthetized animal was also found unfavorable to the appearance of the GABA-induced paroxysmal discharge. In the experiment shown in Fig. 3, arousal was effected by applying high frequency

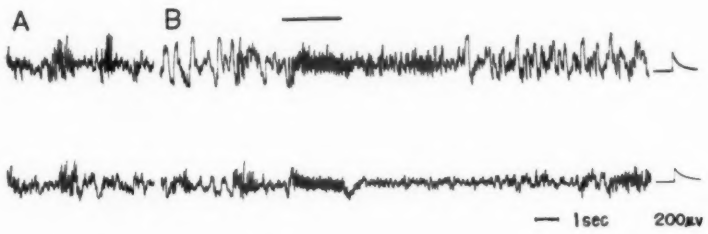

Fig. 3. Suppression of GABA-induced paroxysmal discharges during EEG arousal. Upper and lower records came from GABA-treated and untreated cortical point respectively. A & B, before and after application of GABA. In B, horizontal bar above records indicates approximate duration of reticular formation stimulation. Stimulus parameters, 0.1 msec pulse, 3.5 volts and 300 cps.

electrical stimulation to the mesencephalic reticular formation through electrodes placed with the aid of a stereotaxic instrument. As will be seen from the control record obtained from the untreated cortical point (lower lines in Fig. 3), reticular formation stimulation resulted in suppressing the spontaneous spindling and evoking low-voltage, fast activity, thus producing the activated EEG. On the other hand the recording channel connected to the GABA-treated cortical point clearly showed that the pre-established paroxysmal discharges were completely abolished during EEG arousal and that they recovered hand-in-hand with the reappearance of the spindle activity (upper lines in Fig. 3). In the same way, spontaneous arousal or arousal due to peripheral painful stimulation was proved to be effective in suppressing the GABA-induced paroxysmal discharge.

From these experimental results it can be said that in order for topically applied GABA to develop the paroxysmal activity to a maximal extent, the animal must be put in a state between deep anesthesia and complete arousal. Due to this circumstance, we often encountered great difficulty in producing and maintaining the anesthetic level most suitable for the purpose of the present study. In most of our experiments, however, such difficulty was overcome by

using a short-acting barbiturate, such as thiopental sodium, in an amount as small as possible.

3. *Properties of the GABA-induced paroxysmal discharge*

Several properties of the paroxysmal activity induced by topical application of GABA were investigated and were compared with those of the spontaneous spindling.

i. Effects of topical application of KCl. KCl of 1% in a physiological saline solution was applied to the cortical point which maintained the paroxysmal activity due to a pretreatment with GABA (Fig. 4, A & B). Shortly after application of KCl the paroxysmal discharges were considerably reduced (Fig. 4,

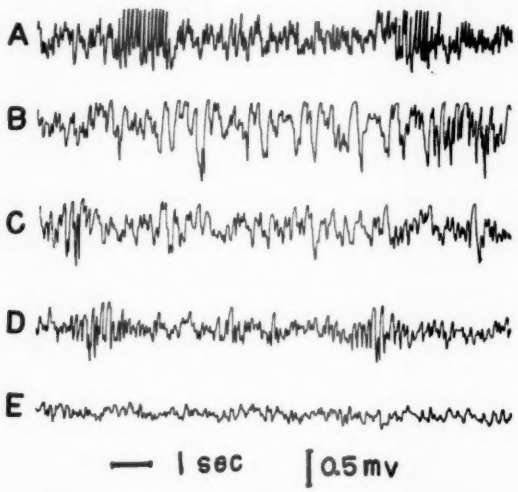

Fig. 4. Effect of KCl upon GABA-induced paroxysmal activity. A & B, before and 13 seconds after application of GABA. C, D & E, 10, 50 and 180 seconds after KCl-treatment. Paroxysmal activity disappeared prior to spontaneous spindle activity.

C), and one minute later, they almost completely disappeared (Fig. 4, D). At this time there were still occurring the phase-inverted spindlings, which, however, disappeared in three minutes after applying KCl (Fig. 4, E). This fact may suggest that the GABA-induced paroxysmal discharge is one type of electrical activity probably independent of the spontaneous spindling.

ii. Occlusion of the common carotid arteries. With a cat showing the well-developed paroxysmal discharges and the phase-inverted spindlings due to a local treatment of the cortical surface with GABA, effects of occlusion of the common

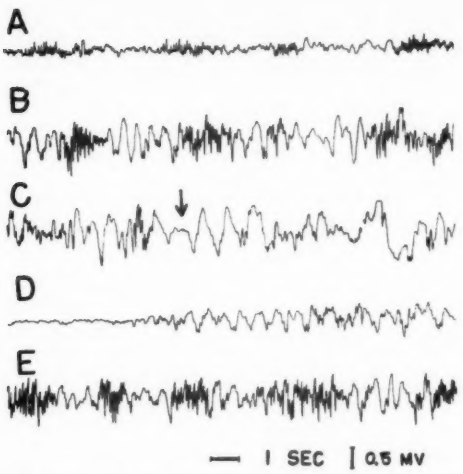

Fig. 5. GABA-induced paroxysmal activity under circulatory arrest. A & B, before and after GABA-treatment. C, the carotid arteries were occluded at arrow. While paroxysmal activity remained unaffected for several seconds after arrow, spontaneous spindlings were promptly abolished. D, 10 seconds after reopening the carotids. Slow waves appeared prior to spindle activity. E, 90 seconds in course of recovery.

carotids were examined. On occluding the carotid on both sides, the phase-inverted spindlings were promptly suppressed, with the paroxysmal discharges almost unaltered at least for a short while after the commencement of circulatory arrest (Fig. 5, C). Continuing the circulatory arrest for about fifty seconds, both kinds of electrical activity were eventually abolished. When the carotids were reopened, the recovery of electrical activity was started within about twenty seconds (Fig. 5, D). The course of the recovery was remarkable in that slow waves with characteristics of the GABA-induced paroxysmal activity preceded the spontaneous spindlings. The complete recovery of both kinds of electrical activity was attained about ninety seconds after reopening the carotids (Fig. 5, E). The fact that the tolerance to hypoxia or anoxia produced by the circulatory arrest is different between the paroxysmal discharge and the spontaneous spindling may again support our view that the neuronal mechanism involved in potential production may be different between the two types of electrical activity.

iii. Destruction of the rostral thalamus. It is a well-known fact that when a rostral part of the thalamus is destroyed, the spontaneous spindling of the frontal cortex suffers a great reduction. This fact has been taken as evidence for a general notion that the spontaneous electrical activity of the cerebral cortex is produced, if not solely, by ascending impulses originating in the subcortical

structures such as the thalamic reticular system.[7] The experiment to be described here was aimed at determining whether or not such notion could also be applied to the spontaneous paroxysmal discharge produced by GABA on the cortical surface.

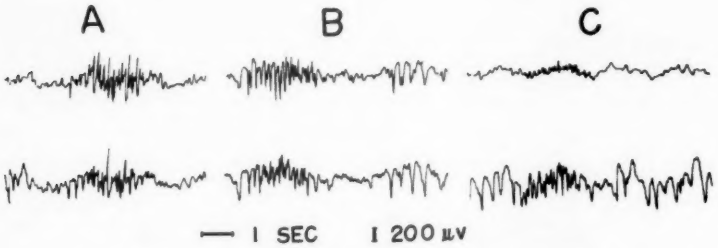

Fig. 6. Abolition of GABA-induced paroxysmal activity by thalamic lesion. Simultaneous records from both sides of the frontal cortex. A & B, before and after application of GABA. After obtaining B, a rostral part of the thalamus was destroyed on one side. The frontal cortex ipsilateral to thalamic lesion (top line) ceased to show marked activity.

In the experiment shown in Fig. 6, recordings were made from two symmetrical points on both sides of the frontal cortex. First, it was ascertained that topically applied GABA exerted its action to about the same extent on both sides of the frontal cortex (Fig. 6, A & B). Then, a part of the thalamus, including the rostral pole of the nucleus reticularis and the nucleus ventralis anterior, was destroyed on one side with the method of electrolytic coagulation with electrodes oriented stereotaxically. This part of the thalamus has been proved to be the origin of the fibers carrying impulses to the frontal cortex from the thalamic reticular system.[8] Immediately following the thalamic lesion, it was found that not only the spontaneous spindling but also the GABA-induced paroxysmal discharge was almost completely abolished in the frontal cortex ipsilateral to the thalamic lesion (Fig. 6, C).

This experiment may offer considerable support for the supposition that as is the case with the spontaneous spindling, the rostral pole of the thalamus serves as an origin of the fibers triggering the paroxysmal discharges in the frontal cortex affected by GABA. However, the dependency of the GABA-induced paroxysmal activity upon the subcortical structures must be considered not to be absolute, because Echlin and Battista[9] found in a chronic experiment that the cortex of the monkey, though deprived of subcortical connections, responded to topically applied GABA with large, rhythmic slow waves which are considered as belonging to the paroxysmal type of activity.

iv. Pyramidal efferent discharges in relation to electrical activity of the

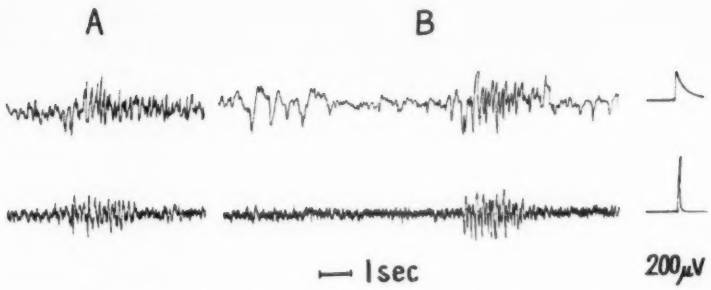

Fig. 7. Electrical activity of the motor cortex (top lines) and pyramidal efferent discharges (bottom lines). A & B, before and after applying GABA to the motor cortex. No marked efferent discharges occurred in association of paroxysmal activity of the motor cortex.

motor cortex. Adrian and Moruzzi[10] were the first who observed the pyramidal efferent discharges waxing and waning in a close synchrony with the spontaneous spindle activity of the motor cortex. This can easily be confirmed, as is demonstrated in Fig. 7 where recordings were made simultaneously from the medullary pyramid of one side and its corresponding motor cortex. In this case the pyramidal efferent discharges were picked up by inserting a fine needle electrode into the medullary pyramid exposed by a ventral approach (Fig. 7, A). In connection with this fact we tried to determine relationship between the GABA-induced paroxysmal discharge of the motor cortex and the pyramidal efferent discharge. Though several efforts were made, there were found no marked efferent discharges of the pyramidal tract occurring in association with the paroxysmal activity produced by GABA on the motor cortex. In contrast with this, the spontaneous spindling, though inverted in phase by the action of GABA, never failed in being associated with the pyramidal efferent discharges. An example of the records showing this feature is reproduced in Fig. 7, B.

DISCUSSION

Many reports on the effects of GABA upon electrocortical activity have been concerned mainly with the suppressing action upon the sharp negative wave of 10–30 msec in duration, both evoked and spontaneous. However, a few papers have been published suggesting that GABA acts to produce the cortical paroxysmal activity besides suppressing the sharp negative wave. Actually one of us (K. I.) noted in a collaborative study with H.H. Jasper that GABA, applied to the cortex of cats with partial destruction of the brain stem, sometimes induced "slow waves similar to those seen in EEG during sleep". Working with succinylcholine-paralysed, unanesthetized cats, Purpura and his associates[11]

described the action of GABA as being "to slow the rate and to increase the amplitude of spontaneous electrical activity". As mentioned previously, Echlin and Battista observed "large, rhythmic slow waves" when they treated with GABA the chronically neuronally isolated cortex of the monkey. According to Takahashi and his associates[5], the GABA-treated cortex of unanesthetized rabbits showed "positive waves" which so far as judged from their records, were as slow as 1 to 3 cps. Though different terms were used for expressing the potential pattern, the electrical activities described by these workers may probably be the same one, belonging to the category of paroxysmal activity. It seems that the paroxysmal activity as referred to in the above-cited papers is the one in which only the slow wave components are strongly activated, with the spiky ones developed very little. A typical example for this type of the paroxysmal activity has been illustrated in Fig. 1, C. At any rate, it is to be noted that the success of the above-mentioned workers in producing the cortical paroxysmal activity was attained by avoiding the use of deep barbiturate anesthesia. Concerning this point, the present experiment has firmly established that the anesthetic level must be adjusted properly in order to develop the GABA-induced paroxysmal activity to a maximal extent.

In addition to the case in which the slow waves having no marked spiky elements appeared upon topical application of GABA, we observed the case too, in which GABA induced electrical discharges characterized by the positive spikes. When the effects of ω-aminoacids were systematically studied by Purpura and his associates[11], the capability in producing the spiky paroxysmal discharge was ascribed not to GABA, but to ω-aminoacids having more than six carbon atoms, such as ε-aminocaproic acid (C_6) and ω-aminocaprylic acid (C_8). As mentioned in the above section, the only effect ascribed to GABA by these workers was the provocation of the slow waves which supposedly had no conspicuous spiky elements. Though we can not explain the discrepancy between Purpura's results and ours, it seems conceivable that as a member of the series of ω-aminoacids, GABA is similar to C_6 and C_8 in being able to induce the paroxysmal discharges of spiky form under favorable conditions. However, it must be admitted that the action of GABA in this respect may be much weaker than that of C_6 or C_8.

The fact that the GABA-induced discharge, either spiky or wavy in configuration, is easily suppressed by arousal stimulation and by central depressant action of barbiturate bears a close parallel to the fact that the strychnine spike tends to be weakened during EEG arousal and in deep barbiturate anesthesia. Furthermore, the positive spike, one of the principal components of the GABA-induced paroxysmal activity, is similar in wave form to the strychnine spike, particularly when the latter is deprived of the negative component by the action of GABA. In the light of these facts, it seems probable that the site of potential production may be the same in the GABA-induced positive spike and the

strychnine spike. Since it is widely accepted that the strychnine spike is produced by the dendrites of the pyramidal cells[12], we presume that the positive spike produced by GABA may also be ascribed to dendritic activity. On the other hand it has been supposed that the spontaneous spindling is due to electrical activity of the dendrites of the pyramidal cells.[13] As has been repeatedly confirmed, this type of dendritic activity is well maintained under the influence of GABA, although its electrical polarity is reversed from negative to positive. As a conclusion of this consideration, the event occurring at the cortex treated with GABA may probably be such that while preserving the spindle activity, the dendrites of the pyramidal cells are simultaneously activated to show the paroxysmal spiky discharges. However, as we have pointed out, there are differences between the spontaneous spindling and the GABA-induced paroxysmal discharge in regard to the time of survival under the action of anoxia and of topically applied KCl, as well as in regard to relationship to the pyramidal efferent discharges. In view of these facts, it is supposed that the spontaneous spindling and the GABA-induced spiky discharge, though both representing dendritic activity, are different from each other in a crucial point, for example, in the mode of activation of the dendrites.

So far we have discussed the origin of the GABA-induced paroxysmal discharge with respect to the spiky element of positive polarity. As we have noted, however, the paroxysmal activity sometimes takes a form of slow waves of 0.5 to 3 cps without any marked spiky concomitant. Though we have been unable to find any marked difference of property between the positive spike and the slow wave, the difference of potential pattern between them is considerable. At the moment there is no experimental evidence available on the basis of which the slow wave can be ascribed to dendritic activity, as has been done with the positive spike. A further experiment seems necessary to elucidate this point.

SUMMARY

The effects of gamma aminobutyric acid (GABA) upon electrocortical activity were studied with lightly anesthetized cats by applying the substance in low concentrations (0.1–1.0%) to the exposed cortical surface. Besides a suppressing action upon the sharp negative wave of 10–30 msec in duration, GABA exhibited an effect of producing high-voltage, spontaneous paroxysmal discharges.

1. The paroxysmal discharges were sustained as long as GABA remained on the cortical surface, and attained several hundred microvolts in amplitude under favorable conditions.

2. The GABA-induced paroxysmal discharges appeared as spike potentials of positive polarity, mostly occurring at a frequency of about 3 cps with or without association of slow waves. Sometimes they were composed of 0.5–3 cps slow waves having no marked spiky deflections.

3. EEG arousal, either spontaneous or produced by stimulation, and deep barbiturate anesthesia strongly suppressed the GABA-induced paroxysmal activity.

4. When KCl was applied to the GABA-treated cortical point, the pre-established paroxysmal discharge disappeared prior to the spontaneous spindle activity.

5. When the common carotid artery was occluded on both sides, the GABA-induced paroxysmal activity showed a stronger tolerance to hypoxia-anoxia produced by the circulatory arrest than the spontaneous spindle activity.

6. An electrolytic destruction of a rostral part of the thalamus of one side resulted in abolishing the pre-established paroxysmal activity at the frontal cortex ipsilateral to the thalamic lesion.

7. There could be found no marked efferent discharges of the pyramidal tract which could be related to high-voltage, paroxysmal discharges produced by GABA on the motor cortex.

We are indebted to Dr. Hajime Okamoto, Professor of Pharmacology of the University of Kanazawa Medical School, who kindly carried out chemical examinations of the sample of gamma aminobutyric acid used in the present experiment. Thanks are due to Dr. Tomoaki Asano for his invaluable suggestions in regard to the manuscript. A part of the expense for this work was defrayed by a grant of the Ministry of Education.

References

1) Iwama, K. & Jasper, H. H., J. Physiol., 1957, **138**, 365.
2) Purpura, D. P., Girado, M. & Grundfest, H., Science, 1957, **125**, 1200.
3) Goldring, S., O'Leary, J. L. & Haung, S. H., EEG Clin. Neurophysiol., 1958, **10**, 663.
4) Caspers, H., Pflüger's Arch. f. ges. Physiol., 1959, **269**, 157.
5) Takahashi, H., Nagashima, A. & Araki, B., Jap. J. Physiol., 1960, **10**, 106.
6) Yamamoto, C., Yuyama, T. & Iwama, K., *ibid.*, 1959, **9**, 160.
7) Bremer, F., Physiol. Rev., 1958, **38**, 357.
8) Hanbery, J., Ajmone-Marsane, C. & Dilworth, M., EEG Clin. Neurophysiol., 1954, **6**, 103.
9) Echlin, F. A. & Battista, A., *ibid.*, 1958, **10**, 766.
10) Adrian, E. D. & Moruzzi, G., J. Physiol., 1939, **97**, 153.
11) Purpura, D. P., Girado, M. & Grundfest, H., Proc. Soc. Exp. Biol., N. Y., 1957, **95**, 791.
12) Bishop, G. H., J. Neurophysiol., 1957, **20**, 255.
13) Clare, M. H. & Bishop, G. H., EEG Clin. Neurophysiol., 1956, **8**, 583.

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Changes in Electrical Sensitivity of the Touch Receptor after a Conditioning Mechanical Stimulus

By

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Motokawa[1] showed that after a photic stimulus the electric excitability of the human eye as tested with an electrical phosphene as an index becomes supernormal for several seconds. The present investigation was undertaken to see if a similar phenomenon would take place with the touch receptor of the human skin.

METHODS

After the epiderm corresponding to a tactile spot had been removed the skin was covered with a film of collodium and a pinhole was made just corresponding to the tactile spot. The stimulating current was made to flow through this pinhole for isolated stimulation of the tactile spot.

The stimulating electrodes used consisted of a bundle of constantan wires which was placed upon the surface of the skin, electrical conductivity being mediated with electrode paste. The area of contact of the electrode was 2 mm. in diameter. A block of metal as an electrode proved to be inconvenient, because air bubbles were formed at the end of the electrode when it was immersed into the electrode paste. Such bubble formation could be avoided by the use of an electrode consisting of a bundle of fine wires. An indifferent electrode was a silver plate 3×5 cm^2 in size held in place on the forearm.

A mechanical stimulus was applied to the tactile spot by means of the electrode mentioned above. It was attached to an iron core of a solenoid. When an electric current was passed, the core was pulled down by electromagnetic action so that the electrode attached to it was pressed against the tactile spot. The strength of the mechanical stimulus could be controlled by the current of the solenoid. The relation between the current of the solenoid and the resulting pressure was investigated with a torsion balance.

The circuit for electrical stimulation is illustrated schematically in Fig. 1. The electric contacts, K_2 and K_1, were opened successively at an interval of 1

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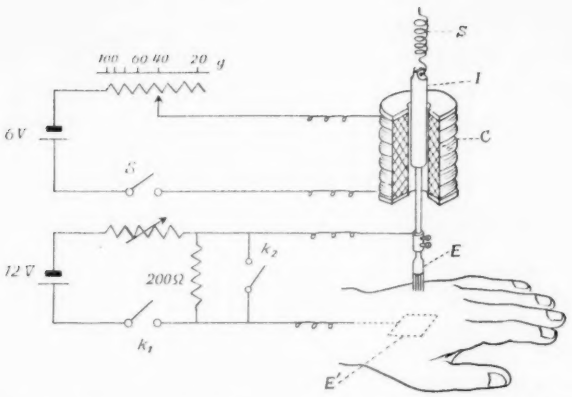

Fig. 1. Arrangements for mechanical and electrical stimulation of mechanoreceptor of human skin. E: Electrode for electrical stimulation. It served to press skin when pulled down by means of an electromagnet. C: Coil of solenoid. I: Iron core of magnet. K_1 and K_2: Electric contacts. S: Spring to suspend iron core. Variable resistance: $0 \sim 10^4 \Omega$.

msec. by a pendulum so that a rectangular pulse of 1 msec. in duration was applied to the mechanoreceptor. The stimulating voltage was controlled by a variable resistance.

A tactile spot at the back of the left hand was used. The forearm was fixed with a supporter of plaster cast. The electrical threshold was measured in such a manner that the intensity was raised in steps from a subthreshold strength. The duration of the mechanical stimulus and the interval between the end of the mechanical stimulus and the electric one were controlled manually following beats of a metronome.

The electrical threshold was found to be lowered temporarily after a mechanical pressure. In another word, the electrical sensitivity of the mechanoreceptor was raised following a mechanical pressure. The degree of increase in sensitivity was expressed quantitatively by the following expression:

$$\frac{\text{Threshold before pressure} - \text{that after pressure}}{\text{Threshold before pressure}} \times 100\%.$$

RESULTS

1. *Time-course of electrical excitability of a mechanoreceptor after mechanical stimulation*

An example of the time-course of electrical excitability of a mechanoreceptor after a mechanical stimulus has been applied is shown by the uppermost curve in Fig. 2. The electrical sensitivity increases above control and reaches a

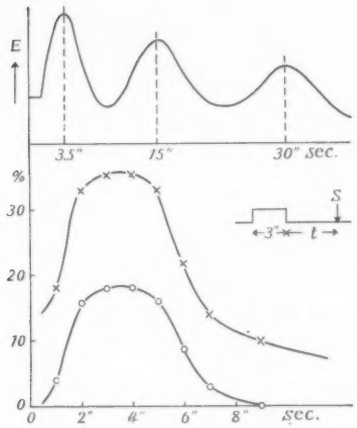

Fig. 2. Time-course of electrical excitability of mechanoreceptor after mechanical stimulation.

maximum about 3.5 sec. after the end of the mechanical stimulus. A second maximum appears at about 15 sec., and a third one at about 30 sec. Such an oscillatory time-course was always observed irrespective of the strength and duration of the mechanical stimulus.

The detailed course of the first maximum was investigated on two different mechanoreceptors. The results obtained are shown in the lower two curves in Fig. 2.

2. *The duration of the mechanical stimulus and electrical excitability thereafter*

In the preliminary experiments it was confirmed that the first maximum of electrical excitability appeared always at about 3.5 sec. Therefore the value of electrical excitability at this moment alone was measured in the following experiments. The duration of the mechanical stimulus ranged from 0.5 sec. to 3.5 sec. The intensity was fixed at a certain value. The results obtained with two different mechanoreceptors are illustrated in the upper diagram of Fig. 3. As can be seen in this diagram, the electrical excitability increases almost linearly with the duration of the conditioning mechanical stimulus, up to a duration of about 2.5 sec. and then at a lower rate. In these experiments the intensity of the mechanical stimulus used was 120 g. per area of 2 mm. in diameter.

3. *The strength of the mechanical stimulus and electrical sensitivity*

The strength was graded between 20 and 120 g. per area of 2 mm. in diameter. The duration of the stimulus was fixed at 3 sec. The electrical sensitivity was measured 3.5 sec. after the end of the mechanical stimulus. The results

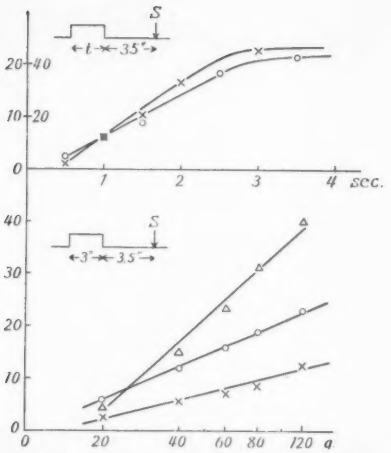

Fig. 3. Effects of duration of mechanical stimulus (above) and of its intensity (below) on electrical excitability of mechanoreceptor.

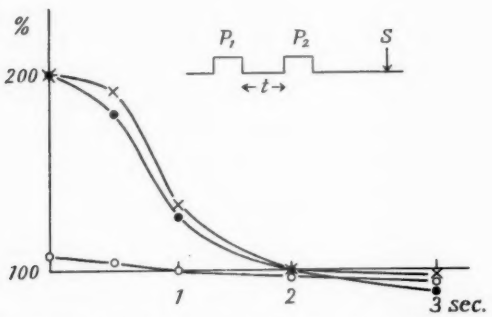

Fig. 4. Summation of two mechanical stimuli (percentage increase of electrical excitability above control) as a function of interval between two stimuli.

obtained with three different mechanoreceptors are illustrated in the lower diagram of Fig. 3. In each receptor the electrical sensitivity increases linearly with the strength of the mechanical pressure. But the rate of increase with pressure is different from receptor to receptor. Such differences may partly be due to individual differences of receptors, but also due to the situation that the mechanical contact between the electrode and the receptor is very difficult to control.

4. *Summation of mechanical stimuli*

Two mechanical stimuli 1.5 sec. in duration and 40 g. in intensity were ap-

plied at various intervals and the electrical excitability was measured 3.5 sec. after the end of the second mechanical stimulus. The results obtained from two receptors are illustrated in Fig. 4. As can be seen in this figure, summation takes place when the interval remains within one second. Two stimuli applied at an interval longer than 1 sec. cannot be distinguished from one stimulus so far as their after-effects on electrical excitability are concerned.

5. *Distant effects of pressure*

In the above experiments the mechanical stimulus was applied directly to the receptor whose electrical excitability was measured thereafter. In the following experiments the mechanical stimulus was applied to points variously distant from the receptor to be tested on its electrical sensitivity. The results are illustrated in Fig. 5. In the inset of this figure points at which mechanical

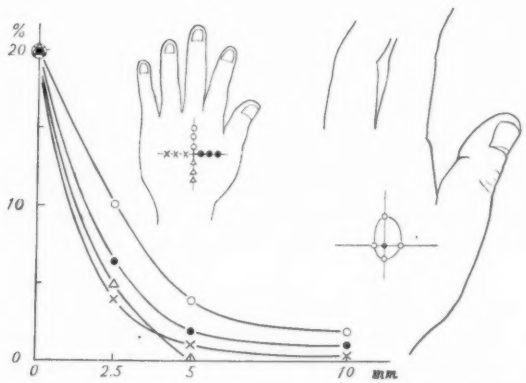

Fig. 5. Distant effects of pressure on electrical excitability of mechanoreceptor. Ordinates: Increase in electrical excitability 3.5 sec. after termination of mechanical stimulation. Abscissae: Distance between receptor and point of pressure application. Further explanation in text.

stimulation was made are marked by solid circles, open circles, triangles and crosses. The curves of electrical excitability measured at 3.5 sec. plotted as a function of distance from the receptor indicate how the effect declines with distance. The rate of decrease depends on the direction from the receptor to the point to which the mechanical pressure was applied. The effect is greatest in the direction towards the fingers or distal direction, and slightest in the direction towards the arm or proximal direction. In the right inset the receptor is represented by a dot, and the distance between this solid and each open circle is made proportional to the value of electrical excitability at a distance of 2.5 mm.

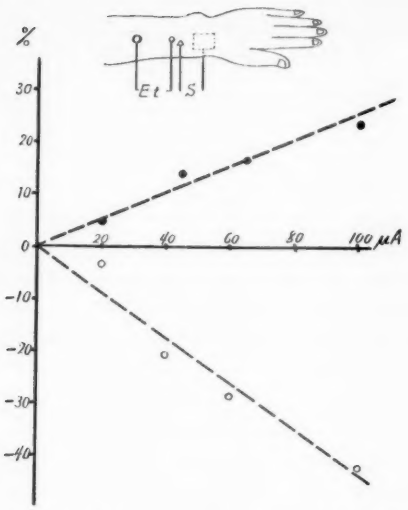

Fig. 6. Effects of electrotonus on electrical excitability. S: Stimulating electrodes. Et: Electrodes for electrotonus. Increase in electrical sensitivity due to catelectrotonus (above) and the decrease due to anelectrotonus (below) are plotted against intensity of polarizing currents.

in respective directions. Whether this effect is due to irradiation of skin sensations or due to deformation of the skin is not clear at present.

6. *Effects of electrotonus*

The effect of electrotonus on the electrical excitability of the mechanoreceptor was investigated. The arrangement of electrodes is shown in the inset of Fig. 6. In this figure, S was electrodes for testing electrical excitability and Et those for applying electrotonus. The distance between S and Et was 2.5 mm. Et consisted of Ag-AgCl electrodes. S and Et were carefully insulated from one another on the surface of the skin so as to avoid direct mutual influences. When the electrode of Et close to the test electrode was made cathodal the electrical excitability increased in proportion to the polarizing current. On the contrary, it decreased linearly with the current of electrotonus when the electrode was made anodal (see Fig. 6).

7. *Effects of local anesthesia*

Local anesthesia with 'Chlorethyl' was made at a distance of 5 mm. from the receptor. The result is shown in Fig. 7. During anesthesia the electrical excitability was so low that the highest voltage gave rise to no response. The

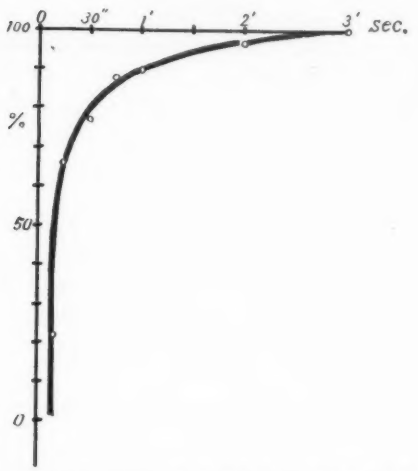

Fig. 7. Recovery curve of electrical excitability after spray of 'Chlorethyl' of area 5 mm. distant from receptor.

electrical excitability during recovery is expressed in percentage of control in Fig. 7 in which a complete recovery is seen in 3 sec. The recovery time was so long with other anesthetics that no systematic investigation was carried out.

DISCUSSION

The long-lasting change in electrical excitability of the eye induced by a light stimulus has been utilized for studies of visual physiology by Motokawa and his collaborators[2-3], but its mechanism has not yet been clarified. It was supposed by Motokawa[1] that some photochemical product may be responsible for such a long-lasting change in electrical excitability. In the present investigation it has been elucidated that a similar long-lasting change in electrical excitability remains after mechanical stimulation of the mechanoreceptor of the human skin. It is not known that any chemical product should be produced at the mechanoreceptor when excited mechanically, but it is apparent that there is no substance to be compared with visual pigments in the eye. Therefore it seems unlikely that the long-lasting change would be due to some chemical product. Especially the waxing and waning of electrical excitability which can be seen after mechanical stimulation of the mechanoreceptor cannot be accounted for in terms of chemical products, because steady dissipation would be expected in such cases.

SUMMARY

A tactile spot of the human skin was stimulated mechanically and then its electrical sensitivity was measured.

1. The electrical sensitivity was raised and declined with waxing and waning.

2. The sensitivity was maximal about 3.5 sec. after the end of a mechanical pressure. The increase in electrical sensitivity was the greater, the longer and the stronger the mechanical stimulus.

3. Two mechanical stimuli showed summation at intervals less than 1 sec. with respect to their effects on electrical excitability.

4. Pressure applied to points at various distances from the receptor to be tested on its electrical excitability showed effects decreasing with increasing distance. The rate of decline was different according to directions from the receptor to the point of pressure application.

5. Catelectrotonus increased, but anelectrotonus decreased the electrical sensitivity of the mechanoreceptor.

6. Local anesthesia depressed the electrical sensitivity.

References

1) Motokawa, K., Tohoku J. Exp. Med., 1949, **51**, 145.
2) Motokawa, K., J. Neurophysiol., 1949, **12**, 291.
3) Motokawa, K. & Iwama, K., Tohoku J. Exp. Med., 1949, **51**, 135.
4) Iwama, K., *ibid.*, 1949, **50**, 71.

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Mucopolysaccharides and Glucidamins in Bovine Lungs

Second Report: On Hexuronic acid-containing Mucopolysaccharides

By

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In 1949, Utusi[1] isolated from pig lungs an anticoagulative fraction, which was supposed to be a mixture of hexuronic acid-containing mucopolysaccharides. Further investigation was carried out by Tokita[2] in 1957, and he stated that pig lung parenchyma contained ordinary heparin, most probably β-heparin and chondroitin sulfuric acid.

The present paper deals with a similar study with fresh bovine lungs, and the study showed the presence of four hexuronic acid-containing (Goldschmiedt-positive) mucopolysaccharides, namely, α-heparin, β-heparin (chondroitin sulfate B), chondroitin sulfate A and hyaluronic acid.

EXPERIMENTAL

Preparation Procedure

I. *Material.* Fresh bovine lungs were freed from adhering membranes and adipose tissues, trachea, bronchi and blood vessels as much as possible, washed with water, then minced.

II. *Extraction.* a) *Autolysis*: 1.78 kg. of the above brei were homogenized with 3.5 l. of water and with 35 ml. of toluol, then incubated at 36°C for 2 days.

b) *Extraction with saline.* The autolyzed mixture was added with 28.6 g. of sodium chloride and several drops of 20% thymol-alcohol solution, then stirred at room temperature (10°C) for 24 hrs.. After centrifugation, the supernatant was filtered through gauze. The tissue residue was further extracted twice with 0.14 M aqueous sodium chloride by the similar procedure as above and the final residue was washed with water. The mixture (7.7 l.) of the above three extracts and washing, which was brown and turbid, was 1/2 saturated with sodium chloride. After standing in a refrigerator (0°C) overnight, its pH was adjusted

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to 4.2 with 1 N hydrochloric acid and the solution was further kept at 0°C for 12 hrs.. The precipitate (Goldschmiedt-negative), which appeared during standing, was centrifuged off. The supernatant was added with celite (1 kg.) and filtered by suction. The filtrate was neutralized with 1 N sodium hydroxide and then dialyzed in cellophane bags against running water for 3 days. A small amount of precipitate formed during dialysis, which was negative for Goldschmiedt reaction, was discarded by filtration. The resultant red-brown translucent filtrate was condensed under reduced pressure to a thick solution, from which the substance was precipitated with abs. ethanol (1 l.), centrifuged, washed with abs. ethanol and ether, dried in vacuo over anhydrous calcium chloride; yield 79 g..

From two batches of fresh bovine lungs (3.28 kg.), 150 g. of reddish brown powder (Goldschmiedt+, Molisch+, biuret+) were obtained. — **Fr. I.**

c) *Extraction with 10% aqueous calcium chloride.* The tissue residue after the above extraction with saline, was homogenized with 3 volumes of 10% aqueous calcium chloride, and the homogenate was adjusted to pH 8.2 with 0.1 N NaOH, then stirred in a beaker at room temperature (10°C) for 24 hrs.. After filtration through gauze, the tissue residue was further extracted twice by the similar procedure as above and final residue was washed with water. The combined solution of all the extracts and the washing was centrifuged and the supernatant (5 l.) was neutralized to pH 6.8 with 0.1 N HCl and dialyzed for 4 days. Small amounts of precipitate appeared during dialysis were centrifuged off. The supernatant was added with 500 g. of celite and filtered by suction. The clear filtrate was condensed under reduced pressure to a syrup, from which the substance was precipitated with abs. ethanol (500 ml.), washed with abs. ethanol and ether, dried in vacuo over anhydrous calcium chloride. Grey powder (Goldschmiedt+, Molisch+, biuret+) amounted to 35 g. — **Fr. II.**

d) *Extraction with 10% aqueous sodium sulfate.* The tissue residue after the extraction with 10% aqueous calcium chloride was homogenized with 3 volumes of 10% aqueous sodium sulfate, and the homogenate was adjusted to pH 7.0 with 10% aqueous sodium hydroxide, then shaken mechanically at room temperature (10°C) for 2 days, followed by centrifugation and filtration through gauze. The residue was further extracted twice with 10% aqueous sodium sulfate by the same way as above, and final residue was washed with saturated aqueous sodium sulfate at 35°C, then with warm (35°C) water. The combined mixture (7 l.) of the extracts and the washings, which was yellowish-brown and a little turbid, was dialyzed for 3 days. The solution was condensed under reduced pressure to 1/10 volumes, and the precipitate (Goldschmiedt −) formed during concentration was centrifuged off. To the supernatant was added 100 g. of celite and the mixture was filtered by suction at 35°C, the celite on the filter paper was washed with 800 ml. of saturated sodium sulfate solution at the same temperature.

The combined mixture of the filtrate and washing was dialyzed for 6 days. The precipitate appeared during dialysis was discarded by centrifugation and the supernatant was condensed to a syrup, from which the substance was precipitated with abs. ethanol (300 l.), washed with abs. ethanol and dried in a vacuum desiccator over anhydrous calcium chloride. About 28 g. of grey poweder (Goldschmiedt+, Molisch+, biuret+) were obtained. — **Fr. III.**

III. *Digestion with enzymes.* After three steps of the above extractions, the tissue residue was dried in a hot air blower. The dried material was crashed into pieces, then homogenized with a mixture of 2 l. of water and 2 g. of trypsin. The homogenate was adjusted to pH 8.0, added with 20 ml. of toluol, and incubated under occasional stirring at 37°C for 3 days. After centrifugation, the insoluble material was homogenized with a mixture of water (1 l.) and toluol (10 ml.) and the homogenate was adjusted to pH 1.5 with 1 N hydrochloric acid. The material was then digested with 2 g. of pepsin at 37°C for 3 days under occasional stirring and readjusting of its pH to 1.5.

The supernatant obtained after trypsin digestion was neutralized to pH 6.8 with 0.1 N HCl and the solution was condensed to 1/5 volumes, then dialyzed for 3 days. To the solution was added 5 g. of barium acetate and the substance was precipitated with abs. ethanol (500 ml.) from the solution, washed with ethanol and ether and dried; yield 36.3 g. (Goldschmiedt+, Molisch+, biuret#). — **Fr. IV.**

The solution obtained after pepsin digestion was neutralized to pH 6.6 with 1 N NaOH, and then centrifuged. From the supernatant, 30 g. of the white powder (Goldschmiedt±, Molisch#, biuret#) was obtained after several steps of treatments; condensation, dialysis, concentration, precipitation with abs. ethanol, washing with abs. ethanol and ether and drying, in turn. — **Fr. V.**

Any uronic acid-containing substance could not be obtained from the residue of the pepsin digestion.

IV. *Separation, Fractionation and Purification of the substances.*

a) Fr. I. The separation of hexuronic acid-containing mucopolysaccharides from the Fr. I. was tried several times without success.

b) Fr. II. 35 g. of Fr. II were homogenized with 10% aqueous sodium sulfate (250 ml.), followed by shaking at room temperature (10°C) for 24 hrs.. The homogenate was then centrifuged and the insoluble material (Goldschmiedt−, Neuberg-Saneyoshi−) was washed twice with saturated aqueous sodium sulfate at 35°C, then discarded.

The mixture of the supernatant and the washings, which was clear light brown, was dialyzed for 3 days. Small amounts of precipitate appeared during dialysis were centrifuged off. The supernatant was added with celite (100 g.) and filtered by suction and the filtrate was again dialyzed for 2 days. The solution was then condensed, from the condensate the substance was pre-

cipitated with abs. ethanol, washed with ethanol and ether, then dried. Almost white powder (Goldschmiedt±, Neuberg-Saneyoshi±) amounted to 5.3 g.. The powder was homogenized with 60 ml. of 90% (v/v) phenol, and stood for 2 hrs. at room temperature (10°C), then centrifuged. The phenol insoluble material was washed twice with 90% phenol, followed by washing with abs. ethanol until no more phenol odor could be detected, then dried in vacuo over anhydrous calcium chloride. 2 g. of white powder were yielded. The powder was then dissolved in water (10 ml.) and kept in a refrigerator (0°C) overnight. The small amount of insoluble material appeared during standing was centrifuged off. To the clear supernatant was added 20 ml. of 10% (v/v) aqueous benzalkonium chloride (Osban)[3]. After standing for one hr. in a refrigerator (0°C), the precipitate thus formed was collected by centrifugation and washed with 50 ml. of 1% aqueous benzalkonium chloride. The substance was extracted from the precipitate with 100, 100 and 50 ml. of abs. ethanol. The combined ethanol extracts (250 ml.) were added with 4 g. of anhydrous sodium acetate and stood at 0°C overnight. The precipitate obtained by centrifugation was washed twice with 100 ml. of 99% ethanol, which was 1/4 saturated with sodium acetate. The precipitate was further washed with abs. ethanol, then dried. About 360 mg. of white powder were obtained. The powder was dissolved in 15 ml. of water containing 2 g. of sodium chloride. After standing at 0°C overnight, it was deproteinized repeatedly according to the Sevag's procedure. The aqueous phase obtained after centrifugation was dialyzed, followed by condensation, precipitation and washing with abs. ethanol and ether. The product was finally dried. 210 mg. of white powder were obtained.

The products was found to be not homogeneous electrophoretically. Therefore, further fractionation was tried as follows: the substance was dissolved in 30 ml. of 1% aqueous barium acetate, and fractionated by successive addition of ethanol. Only the fraction obtained at concentrations of ethanol between 20% and 30% gave positive color for Goldschmiedt reaction. 56 mg. of this white powder were designated as **Fr. II′**.

c) Fr. III. 28 g. of the Fr. III were homogenized with 200 ml. of 90% phenol, and the homogenate was stood at room temperature (10°C) for 3 hrs.. After centrifugation, the phenol insoluble substance was washed twice with phenol, then with abs. ethanol until no phenol was remained, and dried. 6 g. of white powder were yielded. The substance was again dissolved in water (300 ml.) and the solution was stood at 0°C overnight. Small amounts of flocculent precipitate formed during standing were centrifuged off. The supernatant was added with 60 ml. of 10% aqueous benzalkonium chloride, then kept in a refrigerator (0°C) for one hr., followed by centrifugation. The precipitate was washed twice with 100 ml. of 10% aqueous benzalknoium chloride. The substance was extracted from the precipitate with 200, 200, and 100 ml. of abs. ethanol. The

combined ethanol extracts were added with 8 g. of anhydrous sodium acetate, then the mixture was kept in a refrigerator (0°C) overnight. After centrifugation the precipitate was washed with 200 ml. of 99% ethanol 1/4 saturated with sodium acetate, then dried; yeield 650 mg. (Goldschmiedt ±, Molisch ±, biuret ±). An aqueous solution of this product did not show light absorption at wave-length ranges around 260 to 270 mμ, indicating absence of protein containing aromatic amino acids, of nucleic acids and of benzalkonium in the sample. For the removal of a small amount of proteins, the product was dissolved in 30 ml. of water containing 4 g. of sodium chloride and the solution was shaken with 100 ml. of a chloroform-amylalcohol mixture (4 : 1 by volume). After ten times treatment of the Sevag's procedure, no gel was found on centrifugation of the mixture. The aqueous layer thus obtained was dialyzed against distilled water for 2 days, then condensed to a syrup. The substance was precipitated from the syrup with ethanol containing small amounts of sodium acetate, and washed with abs. ethanol, then dried. The yielded white powder amounted to 500 mg. (Goldschmiedt ±, Molisch ±, biuret −). The product was dissolved in 20 ml. of water (the solution showed pH 7.2). After standing at 30-35°C for 4 hrs., 5 ml. of 25% aqueous barium acetate were added to the solution. The mixture was gradually cooled and kept in a refrigerator (0°C) for 2 days. The precipitate formed during standing was centrifuged down, washed with 25 ml. of 5% aqueous barium acetate solution previously chilled at 0°C, and with abs. ethanol, then dried; yield 79 mg. — **Fr. III-a.** To the combined solution (50 ml.) of the supernatant and the washing of the aqueous barium acetate were added 5 ml. of abs. ethanol. The resulting turbid mixture was placed in a refrigerator overnight, then centrifuged. The precipitate was washed with abs. ethanol and dried. 123 mg. of white powder yielded. — **Fr. III-b.** The ethanol concentration of the above supernatant was increased to 50 % with 45 ml. of abs. ethanol. The precipitate thus fromed was collected by centrifugation and washed with abs. ethanol, then dried; yield 240 mg. — **Fr. III-c.** The supernatant obtained at the 50% ethanol concentration was further added with 50 ml. of abs. ethanol, and the solution was stood at 0°C overnight, followed by centrifugation. The precipitate was also washed with abs. ethanol, then dried. White powder amounted to 18 mg. — **Fr. III-d.**. The substance obtained from the final supernatant did not give positive color for Goldschmiedt and Molisch reaction either.

The results of the tests of these fractions are shown in Table I.

d) Fr. IV. 36.2 mg. of the Fr. IV were homogenized with ten volumes of 1/2 saturated aqueous sodium sulfate. The homogenate kept at 35°C with occasional stirring overnight, then centrifuged. The precipitate was washed with saturated aqueous sodium sulfate at the same temperature. The supernatant and the washing were combined and dialyzed against running water for 5 days. Some precipitates (Goldschmiedt −, Molisch −), appeared during dialysis were

Table I. Qulitative Tests for Fr. III a–d.

	Goldschmiedt R.	Neuberg-Saneyoshi R.	Molisch R.
Fr. III–a	++	+	−
Fr. III–b	++	++	−
Fr. III–c	++	+	±
Fr. III–d	±	−	−

centrifuged off. After condensation of the superantant, the substance was precipitated with abs. ethanol, washed with abs. ethanol and dried; yield 1.4 g.. The product was fractionated with 90% phenol by the same procedure as in the case of Fr. III., 23 mg. of the phenol insoluble substance (Goldschmiedt++, Neuberg-Saneyoshi+, Molisch −) were obtained. — **Fr. IV′.** All other fractions were negative for Goldschmiedt reaction.

e) Fr. V. No Goldschmiedt positive fraction was obtained from the Fr. V. by the same fractionation procedure as applied for Fr. III.

V. *Purification and Conversion to the neutral sodium salt.*

75 mg. of the Fr. III-a were dissolved in 20 ml. of water at 35°C, followed by addition of 1 ml. of saturated aqueous sodium carbonate at room temperature (10°C), and then stood for 1 hr. at 0°C. After centrifugation, the insoluble material was washed twice with 5 ml. of 1% aqueous sodium carbonate. The mixture of the supernatant and the washings was acidified to pH 4.6 with 1N HCl and the substance was precipitated with three volumes of abs. ethanol. The precipitate thus formed was collected by centrifugation and washed with abs. ethanol and dried. The product was again dissolved in 5 ml. of water and the solution was neutralized to pH 7.0 with 0.1 N aqueous sodium hydroxide. The substance was precipitated from the solution with 40 ml. of an ethanol-ether mixture (1 : 1 by volume), collected by centrifugation, washed with 40 ml. of ether and dried. White powder amounted to 60 mg. — **Prep. I.**

All other Goldschmiedt-positive fractions; Fr. III-b, Fr. IV′, Fr. II′ except Fr. III-c and Fr. III-d were treated by the same way as above. Sodium salts of these fractions were designated as Prep. II, IV and V, respectiv[illegible].

For further purification of the Fr. III-c, the substance was dissolved in 25 ml. of 1% aqueous barium acetate and fractionated by the successive addition of ethanol. Only the fraction obtained at 10–20% of ethanol concentrations was

Table II. Yields of the Prep. I–V.

	Yield (mg.)		Yield (mg.)
Prep. I	60	Prep. IV	20
Prep. II	115	Prep. V	50
Prep. III	198		

positive for Goldschmiedt reaction. So this substance was converted to the sodium salt by the same way as above, and the final product was designated

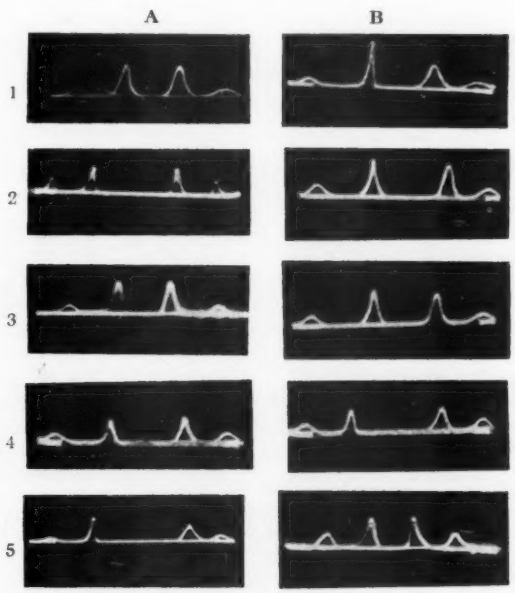

Fig. 1. Electrophoretic patterns of 1% solutions of Prep. I (1), II (2), III (3), IV (4) and V (5) in veronal buffer (pH 8.6, I 0.2) (A) and in acetate buffer (pH 4.7, I 0.2) (B). Temperature 15°C. Current 5 mA.. Exposure 30 min. after starting current. Mobility :

Prep. I pH 8.6 (A1) $\frac{-1.1 \times 0.0132497 \times 0.18}{0.005 \times 1800} = -2.91 \times 10^{-4} \mathrm{cm^2 volt^{-1} sec^{-1}}$

pH 4.7 (B1) $\frac{-1.25 \times 0.0070980 \times 0.18}{0.005 \times 1800} = -1.87 \times 10^{-4} \mathrm{cm^2 volt^{-1} sec^{-1}}$

Prep. II pH 8.6 (A2) $\frac{-0.95 \times 0.0132497 \times 0.18}{0.005 \times 1800} = -2.52$ " "

pH 4.7 (B2) $\frac{-1.12 \times 0.0070980 \times 0.18}{0.005 \times 1800} = -1.68$ " "

Prep. III pH 8.6 (A3) $\frac{-1.0 \times 0.0132497 \times 0.18}{0.005 \times 1800} = -2.65$ " "

pH 4.7 (B3) $\frac{-1.23 \times 0.0070980 \times 0.18}{0.005 \times 1800} = -1.84$ " "

Prep. IV pH 8.6 (A4) $\frac{-1.05 \times 0.0132497 \times 0.18}{0.005 \times 1800} = -2.78$ " "

pH 4.7 (B4) $\frac{-1.3 \times 0.0070980 \times 0.18}{0.005 \times 1800} = -1.94$ " "

Prep. V pH 8.6 (A5) $\frac{-0.95 \times 0.0132497 \times 0.18}{0.005 \times 1800} = -2.25$ " "

pH 4.7 (B5) $\frac{-1.0 \times 0.0070980 \times 0.18}{0.005 \times 1800} = -1.49$ " "

as **Prep. III.**

The yields of these preparations are shown in Table II.

Properties of the Preparations

Electrophoresis. The preparations were examined in both acidic and alkaline buffers. All preparations tested showed one stationary and one migrating boundary within at least 1 hour after starting current as shown in Fig. 1.

Ultracentrifuge[4]. All the preparations examined showed only one boundary as shown in Fig. 2.

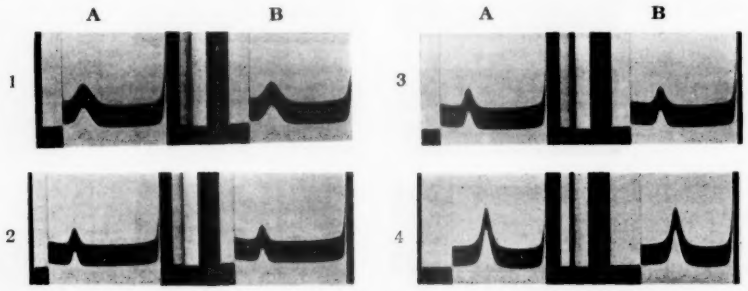

Fig. 2. Sedimentation patterns of Prep. I(1), II (2), III (3) and V (4) (2 mg./ml. solution). Speed of the examination : 59840(I), 59648(2), 59640(3) and 59712 (4) r.p.m., respectively. Migrating from left to right in M/15 phosphate buffer (pH 7.06, I 0.1). Temperature 10°C. Exposure 32 min. (A) and 40 min. (B).

Optical rotation in water.

Prep. I $[\alpha]_D^6 = \frac{0.56 \times 100}{1.02 \times 1} = +54.9°$

Prep. II $[\alpha]_D^6 = \frac{-0.52 \times 100}{0.98 \times 1} = -53.1°$

Prep. III $[\alpha]_D^6 = \frac{-0.23 \times 100}{0.77 \times 1} = -29.9°$

Prep. IV $[\alpha]_D^6 = \frac{0.56 \times 100}{0.99 \times 1} = +56.6°$

Prep. V $[\alpha]_D^6 = \frac{-0.70 \times 100}{1.00 \times 1} = -70.0°$

Anticoagulant activity. The activity was measured by recalcified plasma method according to Reinert & Winterstein[5] and Foster[6], employing heparin sodium salt for investigational use of Upjohn Co. (129 U.S.P. units/mg.) as standard heparin. The extent of clotting in these assays was compared after 1 hr. at 37°C and the amounts of the preparations effecting "50% clot" were calculated. Prep. I, II, IV, V were found to contain 142, 2, 140, 3.3 U.S.P. units/mg. respectively, but Prep. III had no anticoagulant activity.

Test-tube tests. The results of tests with 1% aqueous solution of the preparations are shown in Table III.

TABLE III. Qulitative Tests for the Preparations

Tests	Prep. I	II	III	IV	V
Biuret	−	−	−	−	−
Molisch	−	−	−	−	−
Goldschmiedt	++	++	++	++	++
Neuberg-Saneyoshi	+	++	+	+	+
Ozaki-Turumi					
direct	−	−	−	−	−
indirect	−	±	−	+	+
Bial-Mejbaum for pentose	−	−	−	−	−
Dische for deoxypentose	−	−	−	−	−
Test for P.	−	−	−	−	−
Hydrolyzable sulfur	+	+	+	+	±

Paper partition chromatography. a) Hexuronic acid. 10 mg. of substance were sealed with 1 ml. of 1 N sulfuric acid in a tube and hydrolyzed in a boiling water bath for 15 hours. After cooling the hydrolysate was neutralized with the saturated barium hydroxide solution to pH 6.0, and centrifuged. The sediment was washed three times with 2 ml. of hot water. The supernatant and washings were combined and the mixture was passed through a column (1.0×1.5 cm) of Dowex 50 (H^+) to eliminate barium and hexosamine. The effluent was condensed under reduced pressure below 30°C to dryness and the substance was dissolved in 0.05 ml. of water. Each 0.01 ml. of this solution was sampled separately on three slivers of Toyo Roshi-filter paper No. 3. The one of these slivers was irrigated in a descending way with a mixture of butanol, pyridine and water (5 : 3 : 2 by volume) for 30 hrs. at 23°C; the second with a mixture of butanol, ethanol and water (4 : 1 : 5 by volume) for 18 hrs. at 21°C; and the third with a mixture of n-butylacetate, acetic acid, butanol, methanol and water (3 : 2 : 2 : 1 : 1 by volume) for 12 hrs. at 23°C. Indicator: p-anisidine-phosphate reagent of Mukherjee and Srivostava[7]. The chromatograms of the hydrolysate of Prep. II gave spots corresponding to L-iduronic acid[8]. Those of Prep. III showed a spot corresponding to glucuronic acid and glucuronolactone. The chromatograms of Prep. V showed also a spot corresponding to glucuronic acid and glucuronolactone, but the hydrolysate of Prep. I, whose chromatograms are omitted in Fig. 3, gave no spot at all under these examinations as above.

b) Hexosamine. In each case of Preps. II, III and V, the hexosamine which was absorbed by the column of Dowex 50 above, was eluted with 5 ml. of 1N hydrochloric acid and the eluate was dried under reduced pressure over

solid caustic soda repeatedly, with occasional addition of water. The residue was dissolved in 0.05 ml. of water and each 0.01 ml. of the solution was sampled for chromatography. In the case of Prep. I, 5 mg. of the preparation were hydrolyzed with 1 ml. of 4 N hydrochloric acid[9] in a sealed tube at 100°C for 12 hrs.. The hydrolysate was freed from the hydrochloric acid. And the dried residue was dissolved in 0.05 ml. of water. 0.02 ml. of the solution was sampled for chromatography.

Paper slivers: Toyo Roshi filter paper No. 3. Irrigation: descending way. Solvent: the mixture of butanol, pyridine and dilute hydrochloric acid solution (5 : 3 : 2 by volume, pH 5.5–6.0)[10]. Indicator; aniline hydrogene phthalate reagent of Partridge. The chromatograms of the preparations are shown in Fig. 4.

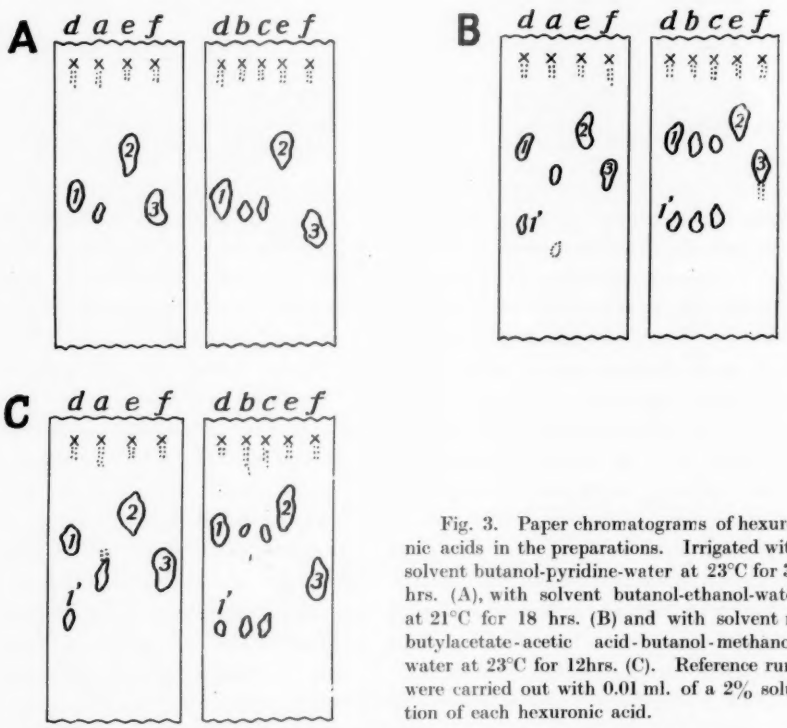

Fig. 3. Paper chromatograms of hexuronic acids in the preparations. Irrigated with solvent butanol-pyridine-water at 23°C for 30 hrs. (A), with solvent butanol-ethanol-water at 21°C for 18 hrs. (B) and with solvent n-butylacetate-acetic acid-butanol-methanol-water at 23°C for 12hrs. (C). Reference runs were carried out with 0.01 ml. of a 2% solution of each hexuronic acid.

a Prep. II; *b* Prep III; *c* Prep. V; *d*, *e*, *f* reference runs; *1* glucuronic acid; *1'* glucuronolactone; *2* galacturonic acid; *3* L-iduronic acid.

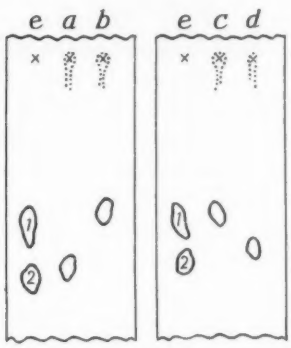

Fig. 4. Paper chromatograms of hexosamines in the Preps. I, II, III and V. Irrigated with the mixture of butanol-pyridine-dilute hydrochloric acid at 23°C for 36 hrs.. Reference runs were carried out with 0.01 ml. of a 2% solution of each hexosamine hydrochloride.
a Prep. I; *b* Prep. II; *c* Prep. III; *d* Prep. V;
e reference run: *1* galactosamine; *2* glucosamine.

Quantitative analyses. The figures are shown in Table IV.

Table IV. Composition of the Preps. I, II, III, IV and V

Analysis	In per cent					In equivalents per equivalent weight				
	Prep. I	II	III	IV	V	I	II	III	IV	V
N*	2.5	2.9	2.8	2.1	3.7	1.5	1.3	1.4	1.2	1.5
Hexosamine†	22.0	27.6	26.2	23.0	30.4	1.0	1.0	1.0	1.0	1.0
Hexuronic acid as glucuronic acid										
carbazole method‡	35.4	17.1	30.1	36.0	32.8	1.5	0.6	1.1	1.5	1.1
mannose-thioglycolic acid method§	13.1	20.9	32.0	—	24.0	0.6	0.7	1.1	—	0.8
Acetyl♯	1.0	7.1	6.8	—	5.0	0.2	1.1	1.1	—	0.7
Sulfur♯	11.0	5.2	5.0	9.9	0.3	2.8	1.1	1.1	2.4	0.1
Ash**	25.1	26.0	20.1	24.0	18.1					
Moisture++	11.2	8.9	6.3	13.4	2.8					

* Micro Kjeldahl. † Masamune and Yosizawa[11]. ‡ Hydrolysis was carried out by 12 hours' heating with 4 N HCl at 100°C (Dische)[12]. § Dische[13]. ♯ Suzuki[14].
** Pregl. ++ The substances were dried to a constant weight by heating in vacuo at 110°C over P_2O_5 for 3–5 hrs..

DISCUSSION

The preparation I from bovine lungs appears to be α-heparin on account of the following findings: 1) the preparation was composed of glucosamine, a hexuronic acid and sulfuric acid in the molar ratio of approximately 1 : 1 : 3; 2) the hexuronic acid in this preparation gave weaker color with mannose-thioglycolic acid and stronger color with carbazole than those for glucuronic acid as reported by Dische & Meyer[15] and Dische[12]; 3) the preparation was remarkably dextrorotatory and had a strong anti-coagulant activity. These characteristics of this preparation coincide well with those of authentic α-heparin. Similar result was obtained also by Tokita[2] with pig lungs.

The present author also showed that the preparation IV as well as the preparation I had a strong anti-coagulant activity. The fact and the analytical data shown in Table IV, indicate that the preparation IV must be α-heparin, too.

The preparation II contained acetylgalactosamine and L-iduronic acid and differed from chondroitin sulfate A and C with respect to the optical rotation and also the solubility in aqueous ethanol. In 1941, Meyer & Chaffee[16] isolated acid mucopolysaccharide of similar sugar components and of similar physical properties from pig skin. Later, Marbet & Winterstein[17] and Meyer & Rapport[18] designated the substance as β-heparin and chondroitin sulfate B, respectively, and found L-iduronic acid in it.[19-21] The preparation II in this paper appears to be β-heparin (chondroitin sulfate B) in comparison of the data in this report with those of previous workers.

The preparation III was consisted of galactosamine, glucuronic acid, sulfuric acid and acetic acid in equimolar proportions, and had specific rotation which was in accord with chondroitin sulfate (chondroitin sulfate A) preparations of several invesitgators.[9,16,22,23,24]

The Prep. V contained glucosamine, glucuronic acid and acetic acid but no sulfuric acid. Anlaytical data together with its optical rotation show that the preparation is identical with the sodium salts of hyaluronic aicd (mucoitin) as those isolated from human umblical cord and from vitreous humor by several investigators.[25-29]

SUMMARY

Four hexuronic acid-containing mucopolysaccharides; α-heparin, β-heparin (chondroitin sulfate B), chondroitin sulfate A and hyaluronic acid (mucoitin) were isolated from fresh bovine lungs.

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References

1) Utusi, Tohoku J. Exp. Med., 1949, **51**, 101, 175.
2) Tokita, *ibid.*, 1957, **66**, 351.
3) Scott's method (Biochim. et biophys. acta, 1955, **18**, 428) was applied by using benzalkonium chloride instead of cetyl pyridinium chloride.
4) Blumberg & Ogston, Biochem. J., 1956, **63**, 715.
5) Reinert & Winterstein, Arch. intern. Pharmacodyn., 1939, **62**, 47.
6) Foster, J. Am. Pharm. Asoc., 1948, **37**, 215, 1947, **36**, 243., J. Lab. Clin. Med., 1942, **27**, 820.
7) Mukherjee & Srivostava, Nature, 1952, **169**, 330.
8) The author is greatly indebted to Prof. M. L. Wolfrom for 1, 2-0-isopropylidene-L-iduronic acid.
9) Wolfrom, Weisblat, Karabinos, McNeely & McLean, J. Am. Chem. Soc., 1943, **65**, 2077.
10) Masamune & Yosizawa, Tohoku J. Exp. Med., 1956, **64**, 53.
11) Masamune & Yosizawa, *ibid.*, 1957, **65**, 169.
12) Dische, J. Biol. Chem., 1947, **167**, 189. & 1950, **183**, 485.
13) Dische, *ibid.*, 1947, **171**, 725.
14) Suzuki, J. Bioch., 1938, **27**, 367.
15) Dische & Meyer, Fed. Proc., 1947, **6**, 248.
16) Meyer & Chaffee, J. Biol. Chem., 1941, **138**, 491.
17) Marbet & Winterstein, Helv. chim. acta. 1951, **34**, 2311.
18) Meyer & Rapport, Science, 1951, **113**, 569.
19) Hoffman, Linker & Meyer, *ibid.*, 1956, **124**, 1252, Arch. Biochem. & Biophy., 1957, **69**, 435.
20) Cifonell, Ludwieg & Dorfman, Fed. Proc., 1957, **16**, 165., J. Biol. Chem., 1958, **233**, 541.
21) Jeanloz & Stoffyn, Fed. Proc., 1958, **17**, 249.
22) Masamune & Ozaki, Tohoku J. Exp. Med., 1943, **45**, 121.
23) Masamune, Yosizawa & Maki, *ibid.*, 1951, **55**, 47.
24) Meyer, Davidson, Linker & Hoffman, Biochim. et Biophys. Acta, 1956, **21**, 506.
25) Suzuki, J. Biol., 1938, **28**, 479.
26) Suzuki, *ibid.*, 1939, **30**, 193.
27) Ozaki & Hasegawa, Tohoku J. Exp. Med., 1943, **45**, 176.
28) Isikawa, *ibid.*, 1951, **53**, 81.
29) Rapport, Meyer & Linker, J. Am. Chem. Soc., 1951, **73**, 2416.

Tohoku J. Exper. Med., 1960, 72, 398–404

Clinical Experience of the So-Called "Atropine Toxicity Therapy (Forrer)"

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Since 1947, Forrer[1-4] has introduced the so-called "atropine toxi ity therapy (A.T.T.)" as a new method of clinical treatment in psychiatry. In the practice of this therapy, at first atropine sulphate in a large dose of 30–200 mg is injected intramuscularly, which introduces a transient disturbance of consciousness as a result of acutely intoxicated conditions lasting about 6–8 hours. For a period of 4–10 weeks, the injection is repeated one to three times per week.

According to Miller[8], Grisell[6], Goldner[5], Schwarz[9] and others, this therapy seems to exhibit certain effects, especially for the patient with tensional state but relatively intact in the ego-structure. However, as even Forrer himself has mentioned, this A.T.T. seems necessary to be more completely established for clinical use, because, according to our experiences, there exist some defects to be reconsidered, especially not only in clinical technique but in effect itself. Paying attention to these points, we have conducted clinical studies of this therapy since 1957. In this paper, the results obtained during the period of our clinical investigations are reported.

SUBJECTS AND METHOD

Besides 2 cases of normal adults who received some basic experiments, we have applied A.T.T. to 51 cases of psychoneurosis and psychosis as shown in Tables I–IV.

In the practice of this treatment, we have employed a similar method to Forrer's principal description, taking care of descriptions on the subjects. Prior to treatment, physical examinations, especially in reference to functional state of visceral organs, were performed, and the cases having no marked abnormalities, including otopharyngeal sphere, were chosen as the subjects in this study.

Usually one hour after light breakfast, atropine sulphate was injected: the

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Table I. Age-distribution of the Treated Subjects with A.T.T.

Age	Male	Female	Total
under 15 yrs.	1	1	2
16 – 20 yrs.	5	6	11
21 – 30 yrs.	12	9	21
31 – 50 yrs.	7	9	16
above 51 yrs.	1	0	1
Total	29	22	51

initial dose was 10 mg in each case, but its dose was increased gradually in the subsequent injections. We marked 220 mg as the largest single dose, but usually the doses ranged 30–50 mg on the average. The injection was performed every other day for a period of 3–7 weeks without cessation. At the initial stage of experiment, atropine sulphate of 0.5 mg solved in 1 ml was injected, but in later stage, that of 5 mg and then 50 mg were applied.

As symptomatic treatment for various disturbances which are apt to occur after the injection of atropine, the following procedures were employed: 1% eserine eye-ointment per 30 minutes for mydriasis; glycerite borax for lipdrying; soft ice-cream for thirst, headache, fatigue, especially for recovery from disturbed consciousness and so on. At the last stage, in order to accelerate recovery from unconsciousness, glucose, Vitamin-B and -C, Pereston-N and Methionine were administered, occasionally adding acetylcholine and methyl-neostigmine sulphate.

CLINICAL RESULTS

1. General View.

Clinical effects in all treated subjects are indicated in Table II, in which "good" signifies complete remission or sufficient improvement to be discharged from hospital, "fair" a slight degree of improvement and "unchanged" little or no effective results. Forrer has made a classification in his report, consisting of 1) aggravation, 2) unchanged, 3) light improved, 4) moderately improved and 5)

Table II. Diagnostic Classification of Subjects and Effects of A.T.T.

Diagnosis		Good		Fair		Unchanged		Total	
Schizophrenia	Acute	3	6	4	14	2	19	9	39
	Intermediate	3		9		6		18	
	Chronic	0		1		11		12	
Manic-depressive Psychosis		1		3		2		6	
Psychoneurosis		2		1		1		4	
Psychopathy		0		1		1		2	
Total		9		19		23		51	

significantly improved, though 1)–4) need more or less the hospital care. It seemed difficult to evaluate our subjects according to such categories, because they consist of various kinds of psychotics, however the cases classified as "good" might accord with 4) and 5) groups in Forrer's description. Also, in our experience, we could find only one case (schizophrenia) which turned to aggravation by application of A.T.T.

The effect in relation to the duration of disease is shown in Table III. There are many improved cases with a duration of less than one year, but more than a half of the cases with a duration over one year account for unchanged group.

Table III. Relation between the Effect and the Duration of Disorders.

Duration	Good	Fair	Unchanged	Total
within one year	7	8	7	22
over one year	2	11	16	29

Many cases reported in this paper were treated by other therapies before application of A.T.T., because they did not show any responses to the routine psychiatric treatment. To such cases A.T.T. was actually applied at least more than one month after cessation of the previous routine therapy. Accordingly, the influence of those previous treatments upon the effect of A.T.T. might be rather denied in our evaluation.

As shown in Table IV, however, the cases treated with A.T.T. are expediently divided into two groups: the one treated with A.T.T. alone and the other treated with additional treatment for a short period immediately after A.T.T. On comparison of these two groups, it is noted that there are some differences especially among the improved cases, since the latter slightly exceeds the former in effect. This means an application of some additional treatments is more desirable than A.T.T. alone, if better improvement is further pursued. This may be regarded as one of the defects of A.T.T. in clinical practice as well.

Table IV. Relation between the Effect and the Therapies Combined with A.T.T. (the Therapy Applied after Cessation of A.T.T. is picked up here). Brackets show Number in Schizophrenic Cases. *Lobotomy had been performed above more than a Half Year in Each Case.

Therapy	Good		Fair		Unchanged		Total	
Electroshock	1(1)	5(4)	2(1)	8(7)	3(2)	9(8)	6(4)	22(19)
Chlorpromazine	3(2)		3(3)		3(3)		9(8)	
Reserpine	—		1(1)		3(3)		4(4)	
Insulin-shock	1(1)		2(2)		—		3(3)	
*Lobotomized cases			2(2)		6(5)		8(7)	
A.T.T. alone	4(2)		11(7)		14(11)		29(20)	

Table II indicates the clinical results of "good" in 18%, "fair" in 37% and "unchanged" in 45%. However, in Table IV, the results in the cases applied A.T.T. alone show 14%, 38% and 48% respectively, though in the group with some combined therapies the results are 23%, 36% and 41% respectively. Although there are certain fluctuations, our estimation of the clinical effect of A.T.T. shows improvement in 15%, fair effect in 35–40% and unchanged or ineffective in 45–50%.

The effect on individual symptoms is as shown in Table V. It is exerted not only on emotional disturbances but on the sphere of perception or thought.

Table V. Effects on Each Symptom Recognized in the Treated Subjects.

Symptom	Effective			Symptom	Effective		
Insight for disease	8	among	45	Depersonalisation	8	among	14
Delusion	16	"	26	Negativism	3	"	10
Spontaneity	9	"	22	Euphoria	7	"	9
Psychomotor excitement	16	"	20	Depressive-Feeling	3	"	5
Hallucination	14	"	18	Stereotyped Behavior	1	"	4
Anxiety	12	"	17	Compulsion or its idea	2	"	3
Incoherence	7	"	15				

The outcome of effects of A.T.T. is revealed early. In general, it begins at the time of 3rd or 7th injection and advances with improvement by repetition of treatment. Conversely, in ineffective cases, with the 10th injection, there scarcely appear any changes. There are several improved cases which have maintained good condition up to now. In addition, the results in each of the above-described Tables were evaluated at the time of more than a half year following A.T.T.

2. Effects in Each Disorder.

Schizophrenia: The results in treated 39 cases are shown in Tables IV and VI. It was more effective in acute cases than in chronic ones, and also in paranoid form than in other types. Usually, in the improved cases hallucinations and delusions decrease gradually, with which a relatively good adaptation to environment tends to be introduced with considerable improvement in the sphere of psychomotor or emotion, accompanying amelioration of abnormal aggitated behavior and restlessness. Especially interesting is that, even if depersonalisation or other schizophrenic symptoms or experiences did not completely disappear, the patient is apt to object his symptoms and subject himself to the reality. Of course this attitude or behavior does not directly relate to obtaining the definite insight, but actually there appears a certain self-insight lacked before application of A.T.T. As shown in Table IV, there were many cases with good effect under combined therapy with A.T.T., excepting all lobotomized cases. Also, in such

chronic cases which had a transiently aggitated state in the past, a good response was in general recognized by A.T.T., as compared with cases lacking in such episodes in anamnesis.

Table VI. Clinical Effects on Schizophrenia

Classification	Good		Fair		Unchanged		Total	
Duration of disease								
within $\frac{1}{2}$ yr.	3	6	4	14	2	19	9	39
$\frac{1}{2}$ – one yr.	2		1		3		6	
1 – 3 yrs.	1		7		3		11	
above 3 yrs.	0		2		11		13	
Clinical Type								
Catatonia	1	6	4	14	5	19	10	39
Hebephrenia	1		8		12		21	
Paranoid	4		2		2		8	

Manic-depressive psychosis: One case diagnosed as essential mania showed complete remission with a 20-times injection of atropine sulphate. Other two cases exhibited a poor response and needed electroshock treatment for several times. Unlike these cases, depressive psychoses, especially involutional depression, did scarcely respond to A.T.T. Accordingly, our impression of A.T.T. in depressive-affective psychosis seemed rather negative in contrast to Forrer's opinion.

Psychoneurosis: There were individual differences, but, in general, it seemed effective in the cases of anxiety, tension, restlessness and phobia. During the course of treatment, almost all cases tended to show a transient aggravation. Therefore it was doubtlessly necessary to add psychotherapies. Nevertheless, the effect on the compulsive state was only transient, because such cases usually went to relapse later.

Other cases: One case which had complained of nervousness and sleeplessness for about 10 years under neurasthenic condition, showed a fairly good response. But one case of psychopathy with weak-will and alcoholic addiction did not show any responses to A.T.T.

COMMENT

At first, to the best of our knowledge, there were no severe side-effects due to A.T.T. itself. We have experienced 756 times of injection in total, with up to 3300 mg of atropine sulphate as the highest value in total dose in one case. This might indicate, at any rate, that A.T.T. is a relatively safe treatment even with a large dose of atropine. Of course the doses used in our study are rather smaller than the lethal dose in animal[10]. However, the subject treated with

A.T.T. usually complains of a severe psychic pain during the course toward unconsciousness or recovery, as well as in case of insulin-shock treatment. In spite of the lessened danger than in insulin-coma, this subjective feeling or complaint might be a defect of A.T.T. in clinical practice.

As regards clinical results of A.T.T., Forrer reported that significant improvement was found in 12% and moderate one in 8%. According to Schwarz, the former was 25% and the latter, 32%. Our results rather resemble Forrer's data. Even though it should not be denied that A.T.T. has certain effect upon psychosis, we are inclined to an opinion that the effect of A.T.T. is somewhat overestimated.

Not only Forrer[4], Schwarz[9] but Grisell and Bynum[6] have demanded that A.T.T. is effective in the case with anxiety and tension but without destruction of ego-structure. In fact, even in our experience, the effect was significant in such cases. However, concerning the ego-structure iteself, our results suggest few explanations, because above a half of our subjects consisted of chronic schizophrenia.

Following A.T.T. many cases tended to obtain a certain self-insight to object himself and to behave realistically to environment. The psychic change with such a mode seems to be different characteristic of A.T.T. from other routine psychiatric therapies. Moreover, even in chronic schizophrenics with enertia, apathia and dementia, somewhat transient psychomotor excitement during the course of A.T.T. was occasionally found, and certain good effects seemed to exert on some of those cases or patients who had once shown aggitation in the past. These findings are interesting in the respect that A.T.T. has some property to have influences upon the deep structure of psychic phenomena.

During the course of falling into unconsciousness after the injection of atropine sulphate, we could recognize a transient occurrence of specific disturbance in perception which closely resembles that introduced by mescaline or LSD, viz. the so-called "hallucinogenics". This condition which might be called a sort of changed consciousness seems to play an important role in A.T.T. for reorganization of ego, since application of psychotherapy was more or less necessary in performance of this therapy. However, besides such effects, the direct action of atropine on the brain itself, such as the reticular formation or the hypothalamic region, must be considered in relation with acetylcholine.[7]

SUMMARY AND CONCLUSION

The results of clinical experience of the so-called "atropine toxicity therapy" applied to 51 cases of various kinds of psychotics were as follows:

1) A good effect such as complete remission was obtained in 6 cases of schizophrenia, one case of mania and 2 cases of psychoneurosis and a moderate effect, in 14 cases of schizophrenia, 2 cases of mania, one case of depression, one

case of psychoneurosis and one case of psychopathy.

2) This therapy which was applied alone was estimated 15% in good effect, 35–40% in fair effect and 45–50% in unchanged : Therefore the effect should not be overestimated.

3) In clinical picture, this therapy is effective for anxiety, tensional state and psychomotor excitement, and then for delusion and disturbance in perception: Almost all patients tend to have insight or to object himself to the reality.

4) Psychic condition in the course of falling into disturbed consciousness resembles that introduced by the hallucinogenic drugs, and also, such a condition might be an important entity in clinical practice, especially for the purpose of application of psychotherapy, besides which the direct influence of atropine on the brain should not be denied.

5) This therapy may be regarded as a relatively safe method, because we found neither severe side-effects nor complications even at the time when 220 mg of atropine sulphate was injected as the largest single dose or the total dose of 3300 mg in one case.

References

1) Forrer, J. Michigan State Med. Soc., 1950, **49**, 184.
2) *Ibid.*, Am. J. Psychiat., 1951, **108**, 107.
3) Forrer & Draper, J. Nerv. & Ment. Dis., 1953, **117**, 226.
4) Forrer, *ibid.*, 1954, **120**, 40; 1956, **124**, 256.
5) Goldner, *ibid.*, 1956, **124**, 276.
6) Grisell & Bynum, *ibid.*, 1956, **124**, 265.
7) Meyers, J. Pharmacol. & Exp. Therap., 1952, **104**, 389.
8) Miller, J. Nerv. & Ment. Dis., 1956, **124**, 260 and 269.
9) Schwarz, *ibid.*, 1956, **124**, 281.
10) Ikeda, Yakubutsu-Chishiryo Shu, Nanzando, Tokyo, 1956. (Jap.)

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Blood Pictures in Stomach Cancer, Especially on Their Relation with Cancer Progress*

First Report: Serum Proteins and Serum Colloidal Reactions

By

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Though many studies have been published concerning the tumor-host relation of malignant neoplasms, most of them are experimental researches on the mechanism and little is known on the clinical relation between the extent of progress of a tumor and the type and grade of general response of the patient having the tumor. In the present series, blood pictures of stomach cancer cases were studied with special reference to their relation with various natures of the tumor, including the extent of progress revealed by operations in our clinic.

The first report presents the results of serum protein analysis and serum colloidal tests. The conclusions of the whole series will appear in the fourth (last) report, with a discussion.

EXPERIMENTAL AND COMMENTS

Serum Proteins

Method

The serum protein analysis was made on 80 cases with stomach cancer (52 males and 28 females; aged 25 to 74 years, 55.0 years on an average), and 20 healthy adults and 28 cases with peptic ulcer of the stomach and/or duodenum for comparison. The concentration of total protein (T.P.) was measured refractometrically, and that of albumin (Al.), α-, β- and γ-globulins (Gl.) electrophoretically after Tiselius.

Results

The serum protein figures in each of the above-mentioned three groups are

* A part of this article was read at the 18th general meeting of the Japanese Cancer Association, Tokyo, 1959.

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shown in Table I. The features rather characteristic of the cancer cases were a remarkable decrease in T.P. and in Al. and an increase in globulins, especially in α-Gl., accompanied with a decreased albumin-globulin ratio (A/G). This coincides with the results of Sato[1] and Kumei[2] in our laboratory, and of many other authors.

Table I. Serum Protein Figures in Stomach Cancer and in Controls

Cases	No. of cases	Mean values in g/dl.					
		T.P.	Al.	α-Gl.	β-Gl.	γ-Gl.	(A/G)
Healthy adults	20	7.19	4.17	0.62	1.07	1.33	(1.41)
Peptic ulcer	28	6.91	3.76	0.72	1.09	1.33	(1.22)
Stomach cancer	80	6.57	3.25	0.83	1.14	1.36	(0.99)

Relation with the extent of cancer progress (Table II). Total protein and Al. were more decreased and α-Gl. was more increased in P.C. II or III cases than in O or I cases, and in L.M. II or III cases than in O or I cases. Thus, the grade of the above-mentioned changes in serum proteins of the stomach cancer cases was found to depend largely upon the stage of progress of the lesion.

Table II. Relation of Serum Protein Figures in Stomach Cancer with Extent of Cancer Progress

Cancer spreads		No. of cases	Mean values in g/dl.					
			T.P.	Al.	α-Gl.	β-Gl.	γ-Gl.	(A/G)
Peritoneal dissemination*	P.C. O, I	34	6.67	3.40	0.77	1.14	1.37	(1.05)
	II, III	46	6.51	3.14	0.89	1.14	1.36	(0.94)
Lymph-nodal involvement†	L.M. O, I	16	6.74	3.45	0.76	1.13	1.39	(1.08)
	II, III	64	6.53	3.20	0.85	1.14	1.35	(0.97)
Stage of progress‡	Stage I	11	6.74	3.54	0.72	1.12	1.36	(1.13)
	II	58	6.64	3.29	0.85	1.16	1.37	(0.98)
	III	11	6.12	2.81	0.86	1.07	1.33	(0.86)

* Superficial spread of stomach cancer via the peritoneum.[3] P.C. O (P.C. is an abbreviation of "peritonitis carcinomatosa"), no aggression to the stomach serosa; I, involvement of only the stomach serosa; II, disseminations to the serous membrane adjacent to the stomach, for example, those to the omenta; III, extensive disseminations showing an inoperable condition so-called carcinomatous peritonitis.

† L.M. O, no metastasis to the lymph nodes; I, metastasis to the stomach nodes; II, those to the retroperitoneal nodes; III, advanced metastasis to the farther nodes which cannot be removed surgically.

‡ Extent of the cancer progress judged from the state of the dissemination and of the metastasis. Stage I, the comparatively early stadium in which both P.C. and L.M. are O or I and no metastasis is found at any organ; II, the intermediate stadium between I and III; III, the inoperable stage in which either P.C. or L.M. is III, or an organ metastasis is recognized.

Relation with morphology of the main tumor (Table III). The concentration of Al. was comparatively low and that of α-Gl. high in cases with the large tumors, suggesting some relationship between the size of the tumor and the serum protein figures of its host. The relations between the serum protein figures and the gross or histological features of the tumors were, on the other hand, very complicated, but no significant relation was found in so far as A/G was concerned.

TABLE III. Relation of Serum Protein Figures in Stomach Cancer with Morphology of the Tumor*

Morphology		No. of cases	Mean values in g/dl.					
			T.P.	Al.	α-Gl.	β-Gl.	γ-Gl.	(A/G)
Size†	Small	14	6.76	3.48	0.79	1.14	1.39	(1.02)
	Medium	17	6.57	3.31	0.79	1.12	1.32	(1.06)
	Large	39	6.62	3.25	0.85	1.16	1.38	(0.97)
Gross appearance‡	Circumscribed	43	6.52	3.25	0.81	1.14	1.33	(1.00)
	Infiltrative	27	6.83	3.46	0.83	1.17	1.41	(1.00)
	Ulcerated	62	6.63	3.32	0.81	1.15	1.36	(1.00)
	Non-ulcerated	8	6.74	3.38	0.88	1.14	1.35	(1.01)
Histological feature	Adenomatosum	25	6.75	3.41	0.89	1.15	1.37	(1.03)
	Solidum	43	6.59	3.27	0.82	1.13	1.37	(0.99)
	Mixed	2						

* In 70 cases which were subjected to resection of the stomach.

† Small, the product of both diameters of the main tumor being below 20 cm^2.; medium, 20 cm^2. or more but less than 40 cm^2.; large, 40 cm^2. or more.

‡ "Circumscribed" includes Borrmann's types I or II; "infiltrative", types III or IV; "ulcerated", types II or III; "non-ulcerated", types I or IV.

Nephelogram[4]

Method

Nephelography, a method to estimate the colloidal stability of the serum, was performed in 55 cases with stomach cancer (37 males and 18 females; aged 25 to 72 years, 53.6 years on an average), as well as in 16 healthy adults and in 21 cases with gastric and/or duodenal ulcer as the controls, by Wuhrmann & Wunderly's original method,[5] as follows.

A series of calcium chloride solutions in physiological saline, at decreasing concentrations from 0.65 to 0.00 per cent, was put in 16 test tubes, to each of them was added 0.1 cc. of the serum and then mixed. They were then boiled for exactly 15 minutes and cooled in water, and then filtered. The filtrates were diluted with three volumes of distilled water, their turbidities being measured photometrically at 550 mμ against distilled water.

In the nephelogram thus obtained (No. of the test tube on the abscissa and

the transmittance on the ordinate), the cardinal values were defined as follows to characterize the curve (Fig. 1) : **a**, the lowest transmittance to show the height of the curve; **b**, No. of the test tube giving the lowest transmittance, to indicate the position of the foot of the vertex; **T**, number of test tubes showing any turbidity, to give the width of the base; and **W**, number of test tubes giving 100 per cent transmittance as the result of a complete coagulation of the serum (W corresponds to the so-called Koagulationsband of Weltmann[6]).

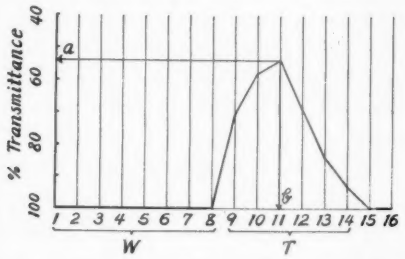

Fig. 1. Schema of a nephelogram explaning the cardinal values.

Results

Table IV shows the results of the nephelography in 16 healthy adults, and Table V those in the ulcer and the cancer cases. The normal range of each value was determined referring to its five per cent rejection limits, in the healthy beings.

In most of the cancer cases a and T increased and b and W decreased remarkably, that is, the lowering and flattening, and the shift to the left of the curve were found to be characteristic of the nephelograms in stomach cancer.

Table IV. Nephelograms in 16 Healthy Adults

Cardinal values	Minimum	Maximum	Mean*	Normal range
a	49	60	54.9±2.0	47 – 63
b	10	13	11.1±0.5	10 – 13
T	5	7	5.5±0.3	5 – 7
W	8	9	8.4±0.3	8 – 9

* Arithmetical means with their 95 per cent confidence ranges.

Relation with the extent of cancer progress (Table VI). It will be noticed that the mentioned characteristic features of the nephelogram in stomach cancer were the more distinct in cases with the more spread lesions.

Relation with morphology of the main tumor. As shown in Table VII, the nephelographic changes in stomach cancer were found to be more marked in

TABLE V. Nephelograms in Stomach Cancer and in Peptic Ulcer

Cases		Distribution (No. of cases)			Mean
		Lower	Normal	Higher	
Peptic ulcer (21 cases)	a	0	13 (61.9 %)	8 (38.1 %)	61.6±2.3
	b	1 (4.7 %)	20 (95.3 %)	0	10.9±0.4
	T	0	16 (76.2 %)	5 (23.8 %)	6.8±0.4
	W	9 (42.9 %)	12 (57.1 %)	0	7.5±0.3
Stomach cancer (55 cases)	a	0	20 (36.4 %)	35 (63.6 %)	64.8±1.3
	b	28 (50.9 %)	27 (49.1 %)	0	9.4±0.6
	T	0	16 (29.1 %)	39 (70.9 %)	8.5±0.7
	W	50 (90.1 %)	5 (9.9 %)	0	5.7±0.8

TABLE VI. Relation of Nephelograms in Stomach Cancer with Extent of Cancer Progress

Cancer spreads		No. of cases	Mean values			
			a	b	T	W
Peritoneal dissemination	P.C. O, I	25	63.6±2.4	10.0±0.1	7.9±0.2	6.4±0.2
	II, III	30	65.7±3.7	8.9±0.3	9.0±0.3	5.1±0.4
Lymph-nodal involvement	L.M. O, I	12	64.8±0.7	9.4±0.6	8.4±0.7	5.6±0.7
	II, III	43	64.7±1.2	9.4±0.4	8.5±0.5	5.6±0.4
Stage of progress	Stage I	7	63.3±3.4	10.0±0.8	7.6±0.7	6.4±0.7
	II	35	64.2±1.7	9.6±0.4	8.3±0.5	5.9±0.5
	III	18	67.1±1.2	8.5±1.0	9.5±1.1	4.5±1.0

TABLE VII. Relation of Nephelograms in Stomach Cancer with Morphology of the Tumor*

Morphology		No. of cases	Mean values			
			a	b	T	W
Size	Small	8	61.4±2.0	10.5±0.6	7.5±0.7	6.9±0.5
	Medium	13	62.9±3.0	9.7±0.2	7.9±0.6	6.2±0.7
	Large	24	65.4±0.6	9.2±0.4	8.7±0.5	5.4±0.5
Gross appearance	Circumscribed	32	64.1±1.5	9.5±0.4	8.5±0.4	5.8±0.5
	Infiltrative	13	64.0±3.6	9.9±0.3	7.8±0.6	6.3±0.8
Histological feature	Adenomatosum	21	64.5±2.3	9.6±0.4	8.1±0.6	5.9±0.5
	Solidum	24	63.5±1.3	9.5±0.4	8.3±0.5	5.8±0.6

* In 45 cases subjected to gastrectomy.

cases with larger tumors but without concern to the macroscopic or microscopic features of the tumor.

Other Colloidal Reactions

Method

The objects were 104 cases with stomach cancer (70 males and 34 females; aged 25 to 74 years, 56.0 years on an average), and 14 healthy adults and 39 cases with peptic ulcer of the stomach and/or duodenum. Tests carried out were the following three:

1) Thymol turbidity test (TTT) by the method of MacLagan.[7]

2) Zinc sulfate test (ZST) according to Kunkel.[8]

3) Sublimate test (ST). To 0.5 cc. of the serum in a test tube 0.25 per cent $HgCl_2$ solution was added at the rate of 20 drops per minute, and the amount of the solution required to make a whity persistent flocculation was estimated, as the ST-value.[9] The test was judged to be positive when this was 0.66 cc. or less.

Results

In Table VIII are presented the numbers of the cases showing the positive results, in each of the tests as well as in each of the cancer and the control groups. The positive ratio of ST was remarkably higher in the cancer cases than in the ulcer cases, though those of TTT and ZST showed no significant differences between the two groups.

TABLE VIII. Serum Colloidal Reactions in Stomach Cancer and in Controls

Cases	No. of total cases	No. of the positive cases		
		TTT	ZST	ST
Healthy adults	14	0	0	0
Peptic ulcer	39	11 (28.2 %)	5 (12.8 %)	11 (28.2 %)
Stomach cancer	104	20 (19.2 %)	14 (13.4 %)	56 (53.8 %)

Relation with the extent of cancer progress (Table IX). ST-positive cases were seen in a higher rate in cases with metastasis to the liver or in those either with marked peritoneal disseminations or with advanced lymphatic metastasis, than in the others, resulting in a close parallelism between the positive ratio and the stage of the cancer progress.

Relation with morphology of the main tumor. The results are shown in Table X. The positive ratio of ST became higher in parallel with an increase of the tumor size, though it showed no distinct relation with the gross or histological features of the tumor.

TABLE IX. Relation of Serum Colloidal Reactions with Extent of Cancer Progress

Cancer spreads		No. of total cases	No. of the positive cases		
			TTT	ZST	ST
Hepatic metastasis	No	95	17 (17.9 %)	13 (13.7 %)	48 (50.5 %)
	Yes	9	3 (33.3 %)	1 (11.1 %)	8 (88.9 %)
Peritoneal dissemination	P.C. O, I	58	9 (15.5 %)	9 (15.5 %)	24 (41.4 %)
	II, III	46	9 (19.6 %)	5 (10.9 %)	32 (69.6 %)
Lymph-nodal involvement	L.M. O, I	26	4 (15.4 %)	6 (23.1 %)	9 (30.8 %)
	II, III	78	16 (20.5 %)	9 (11.5 %)	46 (59.0 %)
Stage of progress	Stage I	21	4 (19.0 %)	4 (19.0 %)	5 (23.8 %)
	II	69	11 (15.9 %)	8 (11.6 %)	40 (48.0 %)
	III	14	5 (35.7 %)	2 (14.3 %)	11 (78.6 %)

TABLE X. Relation of Serum Colloidal Reactions with Morphology of the Tumor*

Morphology		No. of total cases	No. of the positive cases		
			TTT	ZST	ST
Size	Small	28	4 (14.3 %)	3 (10.7 %)	3 (10.7 %)
	Medium	23	5 (21.7 %)	4 (17.4 %)	11 (47.8 %)
	Large	40	6 (15.0 %)	4 (10.0 %)	32 (80.0 %)
Gross appearance	Circumscribed	55	7 (12.7 %)	6 (10.9 %)	29 (52.7 %)
	Infiltrative	36	8 (22.2 %)	6 (16.6 %)	16 (44.4 %)
Histological feature	Adenomatosum	36	5 (13.9 %)	6 (16.6 %)	19 (52.8 %)
	Solidum	53	8 (15.1 %)	6 (11.3 %)	25 (28.3 %)
	Mixed	2			

* In 91 cases subjected to gastrectomy.

SUMMARY

Serum protein analysis, nephelography, thymol turbidity test, zinc sulfate test and sublimate test were carried out in stomach cancer cases operated in our clinic, the results being studied with special reference to their relation with morphological aspects of the lesion.

1) Serum protein figures, the nephelogram and the positive sublimate reaction in stomach cancer were closely dependent upon the grade of cancer spreads.

2) Among various factors concerning morphology of the main tumor, the size was thought to have some relation with serum protein figures and the col-

loidal stability of the serum, while the macroscopic or microscopic features of the tumor had little relation with them.

References and Note

1) Sato, Tohoku Ig. Z. (Jap.), 1953, **48**, 146.
2) Kumei, *ibid.*, 1955, **51**, 577.
3) Muto, 18th General Meeting of International Society of Surgery, Munich, 1959.
4) A more detailed report has been published by Yoshinari, in Tohoku Ig. Z. (Jap.), 1959, **60**, 892.
5) Wuhrmann & Wunderly, Klin. Wschr., 1941, **20**, 564.
6) Weltmann & Paula, Zschr. Klin. Med., 1931, **118**, 644.
7) MacLagan, Brit. J. Exper. Path., 1944, **25**, 234.
8) Kunkel, Proc. Soc. Exper. Biol. Med., 1947, **66**, 217.
9) Hashimoto, M.B.K.P.U. (Jap.), 1950, **2**, 118.

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